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(54) Title: NON-INVASIVE VACCINATION THROUGH THE SKIN (57) Abstract <p>The present invention relates to novel vaccines for the non-invasive, transcutaneous administration of antigens associated with ultradeformable carriers, for the purpose of prophylactic or therapeutic vaccination. The vaccines comprise (a) a transdermal carrier which is a penetrant, (b) a compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity itself, and (c) an antigen, an allergen, a mixture of antigens an/or mixture of allergens. The invention further relates to methods for the vaccination of mammals for obtaining a protective or therapeutic immune response.</p>		

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Noninvasive vaccination through the skin

The present invention relates to novel vaccines for the non-invasive, transcutaneous administration of antigens associated with ultradeformable carriers, for the purpose of prophylactic or therapeutic vaccination. The vaccines comprise (a) a transdermal carrier which is a penetrant, suspended or dispersed in an aqueous solvent in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance, and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, (b) a compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity itself, and (c) an antigen or an allergen. The invention further relates to methods for corresponding therapeutic or prophylactic vaccination of mammals.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art of the present invention. Further incorporated by reference is the complete disclosure content of the co-pending application filed in the name of IDEA AG and bearing the title "Transnasal transport/immunization with highly adaptable carriers".

Skin is the best accessible, but also the most difficult, entry into the body, due to the presence of the stratum corneum. This horny layer of the skin is an evolutionary optimized barrier which resembles the blood vessel wall, in that it comprises flaccid, tightly packed and laterally overlapping cells, whereby the basic cellular-tile motif in the stratum corneum is repeated 20-30 times. The intercellular contacts in the skin, moreover, are sealed with the densely packed and well organized blend of lipids. The stratum corneum therefore not only protects the organism from infections but also precludes an efficient uptake of antigens through the skin. This fact, which is advantageous from the point of allergy, prevented successful immunization or vaccination through the intact skin to date.

The largest drugs on the market in any transdermal delivery device are smaller than 350 Da (Cevc, G. Drug delivery across the skin, *Exp. Opin. Invest. Drugs* (1997) 6: 1887-1937), as only such molecules can cross the tiny, self-sealing pores in the skin. The latter normally are less than 1 nm wide, when hydrophilic, or narrower, when hydrophobic. Organisms such as helminths therefore gain access into the body by penetrating the skin by using their biochemical machinery for the purpose of 'drilling holes' through the organ. Naturally occurring micro-lesions and shunts (such as pilosebaceous units) are available in the skin as well. However, they only cover up 0.1% to 0.5% of the skin surface and, consequently, do not contribute much to transcutaneous transport the fact notwithstanding that bacteria typically exploit such a route for a topical infection (Strange, P., Skov, L, Lisby, S., Nielsen, P. L., Baadsgard, O. Staphylococcal enterotoxin B applied on intact normal and intact atopic skin induces dermatoma. *Arch. Dermatol.* (1996) 132: 27-33.)

Only a few haptens exposed on the skin elicit a cutaneous immune response. This confirms that only sufficiently small molecules from a large load of the topically deposited haptens can find their way into the skin in an appreciable quantity. Such haptens then first irritate the organ and finally may cause hypersensitivity and contact dermatitis (Kondo, S., Sauder, D.N. Epidermal cytokines in allergic contact dermatitis. *J. Am. Acad. Dermatol.* (1995) 33: 786-800; Nasir, A., Gaspari, A. A. Contact dermatitis. Clinical perspectives and basic mechanisms. *Clin. Rev. Allergy and Immunol.* (1996) 14: 151-184). The problem is most serious with the low molecular

weight chemicals or with the pharmaceuticals combined with skin irritants, such as skin permeation enhancers (Cevc, 1997, *op. cit.*). Large molecules seldom are allergenic on the skin, owing to their limited ability to cross the barrier. A Th2 response to a highly immunogenic ovalbumin (Wang, L.-F., Lin, J.-Y., Hsieh, K.-H., Lin, R.-H. Epicutaneous exposure of protein antigen induces a predominant Th2-like response with IgE production in mice. *J. Immunol.* (1996) 156: 4079-4082.) or to Cholera toxin (Glenn, G. M., Rao, M. Matyas, (1998) 391: 851; Glenn, G.M., Scharton-Karsten T, Vasell R, Mallet C.P., Hale T.L. and Alving C.R. Transcutaneous Immunization with Cholera toxin Protects Mice Against Lethal Mucosal Toxin Challenge. *J. Immunol* (1998) 161: 3211-3214.) was possible only after an epicutaneous exposure to a large amount of such proteins and was fairly weak. Moreover, the stratum corneum elimination from the skin was a prerequisite for producing detectable quantities of the specific antibodies against adenoviruses encoding the human carcinoembryonic antigen or human GM-CSF gene in 96% or 43%, respectively, of epicutaneously treated C57BL/6 mice (Deng, H., Qun, L., Khavari, P. A. Sustainable cutaneous gene delivery. *Nature Biotechnology* (1997) 15: 1388-1390.).

No protection against the above mentioned or other epicutaneously employed antigens was reported to date. Antibodies against diphtheria or tetanus toxoid, and bovine serum albumin, which were generated by applying the antigens on the skin of BALB/c mice in combination with cholera toxin (Glenn et al., 1998, *op. cit.*) resulted in a very weak immune response without the adjuvant. Even after the inclusion of Cholera toxin (CT), the average specific antibody titre for diphtheria and tetanus antigens was around 50x and between 70x and 4000x (depending on the inclusion of individual data points), respectively, below that elicited by cholera toxin per se (Glenn et al., 1998, *op. cit.*). The corresponding absolute respective titre values were 14 ± 17 and 8 ± 16 ; the anti-BSA titre was approximately 11 ± 11 (average value \pm standard deviation as calculated from the published figures). No therapeutic or prophylactic effect was demonstrated for these low titres, which shows that the path towards simple non-invasive vaccination is not at all straightforward. The more recent paper published by the same group (Glenn et al., 1998b) demonstrated protection against CT after transnasal challenge which does not allow any conclusion with regard to protection obtainable by transdermal vaccination.

Earlier publications report on the deliverance of proteins across the skin, several orders of magnitude more efficient than in the above mentioned study, as judged by the titres, exploiting mechanosensitive and hydrosensitive, self-regulating carriers (Transfersomes) (for a review, see Cevc, 1997, *op. cit.*). For potent antigens this induced antibody titres that were comparable with those elicited by subcutaneous protein injections: in the case of BSA, the absolute titre of IgG was around 200 in either case (Paul, A., Cevc, G. Non-invasive administration of protein antigens. *Epicutaneous immunization with the bovine serum albumin. Vaccine Res.* (1995) 4: 145-164) and for gap junction protein titres between 15.000 and 100.000 were measured (Paul, A., Cevc, G., Bachhawat, B. K. Transdermal immunization with large proteins by means of ultradeformable drug carriers. *Eur. J. Immunol.* (1995) 25: 3521-3524; Paul, A., Cevc, G., Bachhawat, B. K. Transdermal immunization with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, *Transfersomes. Vaccine* (1997) 16: 188-195.). Yet, the generation of a protective immune response was not demonstrated in either of these publications.

As is known today, the activity of Th1 or Th2 cells plays an important role in immune response: Th1 cells promote mainly the cell-mediated immunity, phagocyte-mediated host defense, but also the production of antigen specific IgG2a in mice. In contrast, Th2 cells tend preferentially to support phagocyte independent host-response, IgG1, IgE and IgA immunoglobulin generation.

The Th1 or Th2 basis of an immune response, that is, the differentiation into Th cell subtypes, not only depends on cytokines and the activity of other regulatory molecules (Luger, T. A., Schwarz, T. The role of cytokines and neuro-endocrine hormones in cutaneous immunity and inflammation. *Allergy* (1995) 50: 292-302; Lohoff, M., Gessner, M., Bogdan, C., Roellinghoff, M. The Th1/Th2 paradigm and experimental murine *Leishmaniasis*. *Int. Arch Allergy Immunol.* (1998) 115: 191-202.); the nature of antigen presenting cells and antigen amount used also play an important role. Cytokines are produced transiently by almost all eukaryotic cells and act via specific cell-surface receptors. Indeed, every cell in the skin, after appropriate stimulation, can release such (glyco)protein factors or express their receptors. Most cytokines are pluripotent and can induce each other or else influence the expression of relevant receptors. This allows cytokines to act in synergistic, additive or antagonistic fashion,

within the framework of so-called cytokine cascade (Luger & Schwarz, 1995; *op. cit.*).

The role of different cells in immunoactivation after cutaneous antigen application is as yet incompletely understood (Luger & Schwarz, 1995; *op. cit.*; Lohoff et al., 1998, *op. cit.*). Langerhans cells, located in the suprabasilar skin region, are believed to play the main role in immunopresentation. These cells first bind and process the antigens, then migrate from the epidermis into the lymphatic vessels, and further into the proximal, draining lymph node, bearing the digested antigens with them. During the process Langerhans cells undergo phenotypical and functional alterations and differentiate into (lymphoid) dendritic cells which finally offer the antigens to naive CD4⁺ T cells that have entered the lymph nodes through the high endothelial venules. In contrast, the other two major types of antigen presenting cells in the skin, macrophages and B lymphocytes, first require activation in order to present antigens and stimulate T cells. Antibodies may be presented to T cells by the venular endothelial cells, and perhaps by certain basic cells of the skin as well.

It is clear, for example, that keratinocytes can augment the local inflammation by producing a plethora of proinflammatory cytokines, including IL-1 α , GM-CSF and TNF α (Pastore, S., Fanales-Belaso, E., Abbanesi, C., Chinni, L.M., Giannetti, A., Girolomoni, G. Granulocyte macrophage colony stimulating factor is overproduced by keratinocytes in atopic dermatitis: Implications for sustained dendritic cell activation in the skin. *J. Clin. Invest.* (1997) 99: 3009-3017). Keratinocyte derived cytokines are also critical for the maturation of Langerhans cells into potent antigen presenting cells (Nasir & Gaspari, 1996, *op. cit.*). The extent to which the former cells directly participate in antigen presentation (Kondo & Sauder, 1995, *op. cit.*) is unknown but the production of inhibitory cytokines, such as IL-10, non-functional IL-12 and TGF β , by keratinocytes is an established fact (Nasir & Gaspari, 1996, *op. cit.*).

The fibroblast pool in the skin also contains cellular subsets that are involved in antigen processing. For example, one subset of fibroblasts is recruited selectively by cytokines at the inflammation site in scleroderma (Fries, K.M., Blieden, T., Looney, R. J., Sempowski, G.D., Silvera, M.R., Willis, R. A., Phipps, R. P. Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis. *Clin. Immunol. Immunopathol.* (1994) 72: 283-292.).

It has been reported previously that epicutaneous antigen application produces a different immune response than the more conventional routes of administration through the oral cavity or the nose. For example, after repeated epicutaneous ovalbumin exposure on the skin anti-ovalbumin IgE-s are prominent (Wang et al., 1996, *op. cit.*). Using bovine serum albumin as a model antigen on the skin, an unusually strong IgA production was previously observed (Paul et al., 1997, *op. cit.*), but no consistent picture of the interdependency between the details of epicutaneous antigen presentation and the resulting immune response emerged to date.

Numerous and different cells participate in mounting an immune response against the cutaneously delivered macromolecules. As has been stated above, the approaches taken so far have not led to the establishment of a convincing strategy for generating a protective immune response. This may be due to the fact that the prior art strategies, such as antigen injection, have not assisted in dissecting the immune response obtainable by applying antigens to the skin to an extent that allows for devising a directed and protective immune response. For example, it is known that antigen injection, as any lesion or other kind of skin perturbation, including the presence of chemical irritants, releases various cytokines from the skin (which not only is the heaviest organ in the body but also makes out the major part of the body immune system). This maximizes the strength, but prevents the fine tuning, of cutaneous immune response, which is also sensitive to the nature of antigens used. High impact vaccine delivery profits from this effect.

Material transport across the skin by means of ultradeformable carriers is just the opposite of said high-impact delivery approach, as it reportedly does not affect the skin. It is believed that this is due to the fact that such hydrosensitive, ultradeformable bodies - so called Transfersomes™ (Cevc, 1997, *op. cit.*), penetrate the stratum corneum through 'virtual channels' between corneocytes, adjusted to the shape of the cells (Schätzlein, A., Cevc, G. Non-uniform cellular packing of the stratum corneum and permeability barrier function of intact skin: a high-resolution confocal laser scanning microscopy study using highly deformable vesicles (Transfersomes). *Br. J. Dermatol.* (1998) 138: 583-592.). It was proposed that Transfersomes push the cells in the skin and intercellular lipids apart during the process, preferentially at the sites of

weakest contact. The passages thus generated seem to be approximately 20-30 nm wide, on the average. They cover several percent (~4%) of the skin surface (Schätzlein & Cevc, 1998, *op. cit.*), the draining of adjacent surface not included. This is much more than the normal shunt area (~0.1%), which explains the quantitative differences between the anti-BSA titres measured after antigen administration with ultradeformable carriers (Paul & Cevc, 1995, *op. cit.*) or by using Cholera toxin as an adjuvant (Glenn et al., 1998a, b, *op. cit.*).

Virtual channels in the skin opened by the carriers appear to be sufficiently wide to let the carriers as well as material associated with them pass through the barrier without significantly perturbing the organ. However, repeated insulin delivery across the skin by means of ultradeformable carriers was found not to induce antibodies against the protein (Cevc, G., Gebauer, D., Schätzlein, A. Blume, G. Ultraflexible Vesicles, Transfersomes, Have an Extremely Low Permeation Resistance and Transport Therapeutic Amounts of Insulin Across the Intact Mammalian Skin. *Biochim. Biophys. Acta* (1998) 1368: 201-215.)

The technical problem underlying the present invention was therefore to establish a means that allows for the successful induction of a medically useful transdermal immune response. The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a transdermal vaccine comprising (a) a transdermal carrier which is a penetrant, suspended or dispersed in an aqueous solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a, preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance, and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration

required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, (b) a compound which specifically releases or specifically induces molecules with cytokine or anti-cytokine activity or exerts such an activity, either of which resulting in the desired, medically useful immune response, and (c) an antigen, an allergen, a mixture of antigens, and/or a mixture of allergens.

As regards the above recited values of up to 99%, it is to be noted that values below 50% of the former relative concentration are often used. Even more advantageously values below 40 rel-% or even around and below 30 rel-% are chosen, whereas with the droplets that cannot be solubilised by the more soluble component relative concentrations that exceed the above mentioned ones by the factor of up to 2 are preferred.

In the context of this invention, the term „pathogen“ refers to an entity which through its presence in or on the body leads to or promotes a pathological state which, in principle, is amenable to or could profit from a preventive, curative or adjuvant immunotherapy. This includes pathogens causing microbial diseases such as extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus*, gram-negative bacteria, such as *Meningococcus* and *Gonococcus* species, species of *Neisseria*, gram negative bacteria, including enteric organisms such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Diphtheria*, *Bordetella Pertussis*, and gram-positive bacteria (e.g. *Bacillus pestis*, *BCG*), particularly anaerobes, such as the *Clostridium* species (e.g. *Clostridium tetani*, *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*); a number of bacteria and all viruses, which survive and replicate within host cells; this latter group encompasses mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes*, retro- and adenoviruses, including but not limited to hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox, (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and various fungi prospering inside host cells; parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks

and mites. The pathogens further include *Brucella* species (e.g. *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae*, *B. ovis*), the causative agent for cholera (e.g. *Vibrio cholerae*), *Haemophilus* species like *H. actinomycetemcomitans*, *H. pleuropneumoniae*, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases. Pathogens in this invention, furthermore, are assumed to include, but are not limited to, the eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body which do not result from microbial infections. Parts of certain pathogens, especially various microbial toxins, have porin-like properties and, consequently, may have some capability to cross the mucosa or to increase the flexibility of penetrant membranes.

The term "specifically" in combination with "releases" or "induces" denotes the fact that the compound interacts with cells capable of releasing cytokines by a receptor-mediated triggering of this cytokine release or induction. This specific release or induction is in contrast to an unspecific release or induction that is, for example, obtained by an intradermal injection.

The term „allergen“ is used in this invention to describe materials of endogenous or xenogenic, e.g., animal or plant, origin which result in an undesired immune response of the body exposed to such an allergen, often resulting in an acute hypersensitivity reaction. Allergising microbes or parts thereof (e.g. of mite), parts of plants (e.g. pollen) or animal (e.g. hair and skin debris), but also man made and inorganic substances belong to this group. On the other hand, nearly any part of the human body, if incorrectly processed by or exposed to the body's immune system, can result in an auto-immune response and lead to the allergic reaction to such a substance. In the narrower interpretation, used when so stated, an allergen is a substance, a group, or an arrangement of substances causing immediate hypersensitivity reactions in the body that could be diminished, or even eliminated, by an immunotherapy, whether done non-invasively through the skin or not.

The term „(therapeutic) vaccination“ in the context of this invention describes any kind of therapeutic immunization, whether done after the disease has been already established, to improve a clinical situation, or else for the purpose of preventing a

disease. Such a vaccination can involve single or repeated administration(s) of the vaccine of the invention. Therapeutic vaccination will either prevent a pathological situation and/or improve a clinical situation. When applied as a preventive agent, it will generally result in a protective immune response.

Immunization denotes any kind of provoking an immune response, irrespective of whether said response is therapeutic or non-therapeutic.

An „antibody“ or an „immunoglobulin“ denotes an IgA, IgD, IgE, IgG, or IgM, including all subtypes, such as IgA1 and IgA2, IgG1, IgG2, IgG3, IgG4. Their „derivatives“ include chemical, biochemical and otherwise obtainable derivatives, such as genetically engineered antibody derivatives. Fragments include, e.g., single chain fragments, Fc-, Fab- F(ab')₂- and other parts of Ig-s, independent of whether they are of endogenous, xenogenic, (semi)synthetic or recombinant origin. Also comprised by the invention are complexes of two or more of the above-recited antibodies, derivatives or fragments.

An „antigen“ is a part of a pathogen or an allergen in its natural form or after fragmentation or derivatisation. More generally, the word antigen denotes a macromolecule or a fragment thereof, any haptenic moiety (for example, a simple carbohydrate, complex carbohydrate, polysaccharide, deoxyribonucleic acid), in short, any molecule recognized by a body's antibody repertoire and possibly capable of antibody induction when administered in the system.

The term "a mixture of antigens and/or a mixture of allergens" means, in accordance with the present invention the combination of at least two antigens or allergens. It is envisaged that also mixtures of antigens and allergens, comprising at least one antigen and at least one allergen, can be used according to the present invention.

The term „cytokine“, as used in the present invention, denotes cytokines, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, with all subtypes, such as IL-1 α and IL-1 β , tumor necrosis factor (TNF), transforming growth factor (TGF- β and - α), Type I and II interferons (IFN- α 1, IFN- α 2, (IFN- ω), IFN- β , IFN- γ), migration inhibitory factor, MIF, c-kit ligand, granulocyte

macrophage colony stimulating factor (GM-CSF), monocyte macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), chemokines, etc., as well as all functional derivatives of any of these molecules.

Cytokines that mediate natural immunity particularly well include type I interferons (IFN- α and IFN- β), tumor necrosis factor (TNF), interleukin-1 (IL-1 α and IL-1 β), interleukin-6 (IL-6) and leukocytes attracting and activating chemokines. The process relies on antiproliferative (e.g. with IFN-s), pro-inflammatory (e.g. with TNF, IL-1) or co-stimulatory (e.g. with IL-6) action, amongst other. Cytokines which best mediate lymphocyte activation, growth and differentiation include interleukin 2 (IL-2), interleukin-4 (IL-4) and transforming growth factor (TGF). Such cytokines, consequently, not only can affect target growth but, moreover, influence the activation of, and thus the production of other cytokines by, the cells which finally may play a role in therapeutic action.

Cytokines that mediate immune-mediated inflammation, which heavily relies on the cell-mediated response, are interferon-gamma (IFN- γ), lymphotoxin (TNF- β , interleukin-10 (IL-10), interleukin-5 (IL-5), interleukin-12 (IL-12) and, probably, migration inhibition factor. Leukocyte growth and differentiation are most affected by interleukin-3 (IL-3), c-kit ligand, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage or granulocyte colony stimulating factor (M-CSF or G-CSF) and interleukin-7 (IL-7).

The term „immunoadjuvant“ is used here to describe any substance which supports, augments, stimulates, activates, potentiates or modulates the desired immune response of either cellular or humoral type, specifically in the case of prophylactic treatment by increasing the antigen specific immune response of any kind and in the case of therapeutic treatment often by supporting cell-mediated immunity. This can be achieved by the addition of suitable cytokines, their blends or antagonists, or less directly by the chemical irritation of the skin, when this contributes directly or indirectly to the release of cytokines from the skin or other involved peripheral tissues, or else by catalyzing or promoting the biosynthesis of the molecules in the tissue which then lead to such action, provided that the final outcome is an increased success of vaccination, that is of prophylactic and/or therapeutic action of used antigen. The class of

immunoadjuvants which indirectly contribute to the useful cytokine pool includes small chemical entities with an allergenic potential, such as certain allergenic (metal) ions, including but not limited to LiCl, HgCl₂, molybdenum, acids, bases and other irritating compounds, such as dicyclohexylmethane-4,4'-diisocyanate, dithiocarbamate (diethyldithiocarbamate), 2,4-dinitrochlorobenzene, isoprinosine, isophorone-diisocyanate, levamisole, (phenyl)oxazolone and alike, Swansonine, sizofran, phthalic anhydride, thymopentin, (fatty) alcohols, (fatty) amines, (fatty) ethers, ricin, or other suitable amphiphiles, many surfactants and chemical skin permeation enhancers, as well as derivatives or combinations thereof; furthermore, (low molecular weight) fragments of or derivatives from microbes, including lipopolysaccharides (such as LPS), cord-factor (trehalose-dimycolate) and other polysaccharides attached to membranes, when used in sufficient quantity, acetylmuramyl-alanyl-isoglutamin, and larger fragments of microbes, including bacterial exo- and endotoxins, or enterotoxins, such as cholera toxin and the heat labile toxin (HLT) of *E. coli*, and their macromolecular fragments, such as A-chain derivatives most, if not all, of which seem to possess ADP-ribosylating activity, the high potency immunoadjuvant LT holotoxin, etc., cell-wall skeleton, attenuated bacteria, such as BCG, etc. Less established examples include clostridial toxin, purified protein derivative of *M. tuberculosis*, LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes*, outer membrane protein of group B *Neisseria meningitidis* (GBOMP), various other peptidoglycans, etc. Immunoadjuvants, in other words, include molecules that alter the uptake or presentation of antigens, activate or increase the proliferation of antigen specific lymphocytes, or interfere with the dominant control mechanism in the immune response, not just in the skin but also in the other immunocompetent tissues. (The mucosal adjuvant activity of ADP-ribosylating bacterial enterotoxins is a well established and known example for this.) On the other hand, molecules which change the (relative) concentrations of cytokines or other immunoadjuvants, such as anti-immunoadjuvant antibodies or other agonists or antagonists of immunoadjuvants, also are immunoadjuvants in the sense of this invention. The same is true for molecules which affect lymphocyte homing, such as various selectins (LECAMS, e.g., various CD62-s), GlyCAM-1, MadCAM-1, VCAM-1, ICAM-1, hyaluronate, etc., and other chemokines, such as RANTES or MCP-1. Endogenous group of immunoadjuvant furthermore comprises histamines, transfer factor, tuftsin, etc. As many of the above mentioned immunoadjuvants do not have sufficient potency to ensure the desired

effect after the non-invasive immunization at too low, and sometimes too high, concentration or on their own, the functional definition of an adjuvant used in this work includes a fortiori sufficient and such modulation of cytokine concentration and distribution pattern in the body that results in mounting the desired therapeutic or prophylactic immune response. If required to gain clarity said modulation and its extent must be determined in a dedicated experiment, in which the specific cytokine levels are determined, for example.

„Immunoadjuvant manipulation“ denotes a non-chemical treatment of the skin, such as skin rubbing, pressing, heating, exposing to an electrical or mechanical, e.g., ultrasound, field, etc., or even an injection of a non-immunogenic formulation in the skin, provided that such treatment releases immunoadjuvant compounds from the skin or other peripheral immuno-active tissues or else reduces the concentration/duration of action of antagonists to the desired vaccination. However, the term „immunoadjuvant manipulation“ also denotes, in accordance with the present invention, a pre-treatment of the skin with immunomodulators and/or cytokines and/or cytokine releasing factors, like, inter alia, histamine.

The term „immunogen“ denotes a hapten coupled to an immunological carrier or an antigen, free or associated with a carrier, which is capable of inducing an immune response.

„Immuno-tolerance“ denotes the lack or, more generally, the reduction of an undesired immune response to an antigen.

Th1 (T-helper cell type I) related antibodies include IgG2a, IgG2b and IgG3.

Th2 (T-helper cell type II) related antibodies comprise the classes of IgG1, IgG4 and IgE.

The term “two forms of a substance” in connection with this invention means two ionization states or salt forms of the same substance, two different complexes of such substance, etc.

„Non-invasive administration“ or „non-invasive delivery“ denotes application on or transport through an intact barrier, in the biological applications dealt with in this disclosure, through intact skin.

„Penetration“ describes a non-diffusive motion of relatively large entities across a barrier. This process typically relies on the penetrant adaptation to the otherwise confining pores in the barrier and also may involve a penetration induced decrease in the barrier resistance, such as pore widening or channel opening; the process does not depend, however, primarily on the penetrant concentration gradient across the barrier.

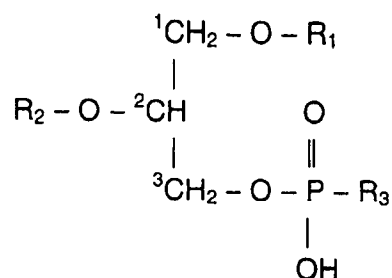
„Permeation“ refers to a diffusive motion across the semi-permeable barriers. The prime example for this is the transport of molecules or molecular aggregates under the influence of a permeating species concentration gradient across the barrier.

A penetrant, consequently, is an entity comprising a single molecule or an arrangement of molecules too big to permeate through a barrier but capable to cross the barrier owing to the penetrants adaptability to the shape and/or diameter of the otherwise confining passages (pores) of a barrier. This adaptability is seen from the fact, for example, that penetrants more than twice bigger than the pore diameter will cross the bilayer without being fragmented down to the pore size. A permeant, on the other hand, is an entity that can permeate through the semi-permeable barrier, such as the skin. A penetrant in an external field experiences a driving force proportional to the nominal penetrant size and to the applied field, which may occur naturally. Such a force, which on the intact, non-occluded skin is believed to originate from the water concentration gradient across the stratum corneum, can result in a penetrant motion through the barrier, including the skin, if the force is strong enough either to deform the penetrant or else to widen the passages in the barrier sufficiently to elude the problem of size exclusion, or both.

For further definitions, especially such pertaining to the penetrants in terms of complex body deformability, the corresponding mechanism of action, lists of interesting penetrant ingredients or selected agents it is referred to the issued or pending patents (DE 41 07 152, PCT/EP91/01596, PCT/EP96/04526, DE 44 47 287). Detailed information relevant for the manufacturing process and penetrant loading with the

antigenic (macro)molecules and/or immunoadjuvants, which are too big to permeate through the barrier, can be found in international patent application PCT/EP98/06750.

Typically, the less soluble amongst the aggregating substances forming a carrier is a lipid or lipid-like material, especially a polar lipid, whereas the substance which is more soluble in the suspending liquid and which increases the droplet adaptability belongs to surfactants or else has surfactant-like properties. The former ingredient, typically, is a lipid or lipid-like material from a biological source or a corresponding synthetic lipid or any of its modifications, such lipid often belonging to the class of pure phospholipids with the chemical formula



where R_1 and R_2 is an aliphatic chain, typically a C_{10-20} -acyl, or -alkyl or partly unsaturated fatty acid residue, in particular, an oleoyl-, palmitoeloyl-, elaidoyl-, linoleyl-, linolenyl-, linolenoyl-, arachidoyl-, vaccinyl-, lauroyl-, myristoyl-, palmitoyl-, and stearoyl chain, and where R_3 is hydrogen, 2-trimethylamino-1-ethyl, 2-amino-1-ethyl, C_{1-4} -alkyl, C_{1-5} -alkyl substituted with carboxy, C_{2-5} -alkyl substituted with hydroxy, C_{2-5} -alkyl substituted with carboxy and hydroxy, or C_{2-5} -alkyl substituted with carboxy and amino, inositol, sphingosine, or salts of said substances, said lipid comprising also glycerides, isoprenoid lipids, steroids, sterines or sterols, of sulphur- or carbohydrate-containing lipids, or any other bilayer forming lipids, in particular half-protonated fluid fatty acids, and preferably is selected from the group of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids, phosphatidylserines, sphingomyelins or other sphingophospholipids, glycosphingolipids (including cerebroside, ceramidepolyhexosides, sulphatides, sphingoplasmalogens), gangliosides or other glycolipids or synthetic lipids, in particular with or else with corresponding sphingosine derivatives, or any other glycolipids, whereby two similar or different chains can be

esterified to the backbone (as in diacyl and dialkenoyl compound) or be attached to the backbone with ether bonds, as in dialkyl-lipids.

The surfactant used, normally, is nonionic, zwitterionic, anionic or cationic, especially a fatty-acid or -alcohol, an alkyl-tri/di/methyl-ammonium salt, an alkylsulphate salt, a monovalent salt of cholate, deoxycholate, glycocholate, glycodeoxycholate, taurodeoxycholate, taurocholate, etc., an acyl- or alkanoyl-dimethyl-aminoxide, esp. a dodecyl- dimethyl-aminoxide, an alkyl- or alkanoyl-N-methylglucamide, N-alkyl-N,N-dimethylglycine, 3-(acyldimethylammonio)-alkanesulphonate, N-acyl-sulphobetaine, a polyethylene-glycol-octylphenyl ether, esp. a nonaethylene-glycol-octylphenyl ether, a polyethylene-acyl ether, esp. a nonaethylen-dodecyl ether, a polyethylene-glycol-isoacyl ether, esp. a octaethylene-glycol-isotridecyl ether, polyethylene-acyl ether, esp. octaethylenedodecyl ether, polyethylene-glycol-sorbitane-acyl ester, such as polyethylenglykol-20-monolaurate (Tween 20) or polyethylenglykol-20-sorbitan-monooleate (Tween 80), a polyhydroxyethylene-acyl ether, esp. polyhydroxyethylene-lauryl, -myristoyl, -cetylstearyl, or -oleoyl ether, as in polyhydroxyethylene-4 or 6 or 8 or 10 or 12, etc., -lauryl ether (as in Brij series), or in the corresponding ester, e.g., of polyhydroxyethylen-8-stearate (Myrj 45), myristate-, -laurate, linoleate-, linolenate-, palmitoleate- or -oleate type, or in polyethoxylated castor oil 40, a sorbitane-monoalkylate (e.g. in Arlacel or Span), esp. sorbitane-monolaurate, -myristate, -linoleate, -linolenate-, -palmitoleate- or -oleate, an acyl- or alkanoyl-N-methylglucamide, esp. in or decanoyl- or dodecanoyl-N-methylglucamide, an alkyl-sulphate (salt), e.g., in lauryl-, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, linolenyl-, linoleoyl-, vaccinyl-, or elaidoyl-sulphate, sodium deoxycholate, sodium glycodeoxycholate, sodium oleate, sodium taurate, a fatty acid salt, with similar preference for aliphatic chains as given above, a lysophospholipid, such as n-octadecylene(=oleoyl)-glycerophosphatidic acid, -phosphorylglycerol, or -phosphorylserine, n-acyl-, e.g., lauryl, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, elaidyl-, vaccinyl-, linoleyl-, linolenyl-glycero-phosphatidic acid, -phosphorylglycerol, or -phosphorylserine, or a corresponding short, double chain phospholipid, such dodecyl-phosphatidylcholine, or else is a surface-active polypeptide. It is important to realize, however, that complexes of polar lipids with other amphipats often can take the role of surfactants in the coating of a carrier and that different ionization or salt states of polar lipids differ widely in their properties. It therefore stands to reason that two different

physicochemical states of the same (polar) lipid mixed together in a membrane will produce a highly deformable carrier satisfying the conditions of this work.

More general information on lipid suspensions can be found in handbook dealing with 'Liposomes' (Gregoriadis, G., Hrsg., CRC Press, Boca Raton, FL., Vols 1-3, 1987), in the book 'Liposomes as drug carriers' (Gregoriadis, G., Hrsg., John Wiley & Sons, New York, 1988), or in the laboratory manual 'Liposomes. A Practical Approach' (New, R., Oxford-Press, 1989). The properties of phospholipids which can be used conveniently to prepare bio-compatible immunopenetrants are reviewed in 'Phospholipids Handbook' (Cevc, G., ed., Dekker, New York, 1995).

It may be convenient to adjust the pH value of a formulation immediately after preparation or just before its application. Such an adjustment should prevent the deterioration of individual system components and/or drug carriers under the conditions of initial pH without sacrificing physiological compatibility. To neutralize a penetrant suspensions it is reasonable to use biocompatible acids or bases to prepare buffers with a pH value between 3 and 12, frequently between 5 and 9 and most often between 6 and 8. Physiologically acceptable acids are, for example, diluted aqueous solutions of mineral acids, such as hydrochloric acid, sulphuric acid, or phosphoric acid, or organic acids, such as carboxyalkane acids, e.g. acetic acid. Physiologically acceptable bases are, for example, diluted sodium hydroxide, suitably ionized phosphoric acid, etc.

If required, immunogen suspension can be diluted or concentrated (e.g. by ultracentrifugation or ultra-filtration) before the application; additives can also be given into the suspension at this time or before. The additives are often selected amongst substances that reduce the formulation sensitivity to ambient stress, including microbicides, anti-oxidants, antagonists of undesired enzyme action, in case cryo-preservants, thickening agents, etc. However, after any system manipulation, the carrier characteristics should be checked and, if required, readjusted.

In accordance with the present invention it was surprisingly found that macromolecular antigens associated with ultradeformable lipid aggregates described herein (immunopenetrants) can cross artificial porous barriers as well as the skin, despite the fact that

the average diameter of said penetrants exceeds the average pore/channel diameter, and that such immuno-penetrants can elicit a therapeutic or prophylactic immune response, provided that said immuno-penetrants are associated with compounds that display cytokine activity or induce the generation in and/or the release of cytokines from the skin, and/or other immunocompetent organs in the body. Alternatively, said compounds antagonize cytokine activity. This latter embodiment advantageously directs the immune response into a Th1 or Th2 dependent immune response by blocking the respective other route. The antigen carriers described in this invention maintain sufficient stability before and during the process. It was further surprisingly found that the resulting immune response is not directly proportional to the applied dose, which implies that the amount of antigen may be varied. Said amount should be well chosen for optimum effect. Choosing optimum amount or range of antigen is well within the skills of the person knowledgeable in the art taking into account the teachings of this specification.

The at least bi-component immuno-aggregates used as carriers in the vaccines of this invention excel in high deformability and most often have the form of vesicles with a highly flexible membrane. Although such carriers have been employed in immunization protocols before, it was unexpected that the previously postulated carrier immunoadjuvancy does not eliminate the need to include compounds with cytokine activity or the appropriate antagonists thereto, preferably IL-4, IL-10, TGF- β , IL-5, IL-6, IL-9 and IL-13; or, after the pre-stimulation of T-cell receptor, also IL-1 in order, to achieve the desired protective immune response. By the same argument, IL-12, IFN- γ and lymphotoxin (LT or TNF- β) are advantageously included to promote the Th1 response and thus to favor the cell-mediated immune response and to provide a means for treating viral and other parasite diseases or for promoting the immunotolerance. For example, a combination of IFN γ , IL-12 and anti IL-4 or simply the addition of IL-12 is expected to revert Th2 response toward Th1 type. More broadly speaking, increasing the relative amount of IL-12 and IL-4 in the beginning of an immune response in favor of the former is proposed to be useful to promote Th1 response also in the case of penetrant mediated immunization, and *vice versa*, whereas IL-2 is going to support NK and B cells growth, to stimulate antibody synthesis, and to affect the magnitude of T-cell dependent immune response in general. Thus, whereas the prior art demonstrated that antibody titres could be

induced by using suitable carriers in combination with antigen and optionally with immunoadjuvants, the immuno responses obtained were not demonstrated to be protective.

A particular advantage of the present invention is due to the fact that it was surprisingly found that the transfer of the penetrant described in this invention does not lead to an essential disturbance of the cytokine composition within the skin. In other words, the transfer of these carriers through the skin will per se not induce any essential release of cytokines. It is therefore possible to study now and to trigger a desired immuno response by including into the vaccine of the invention a compound that specifically induces or releases cytokines from cells in the skin or other organs that are competent to release such cytokines. Fine tuning of a desired immuno response thus may be possible. Alternatively, a compound having or exerting cytokine activity can be included into the vaccine of the invention. Further, an antagonist of cytokine activity may be used that specifically prevents the action of such cytokines. In this embodiment, the immuno response may advantageously be directed towards the Th1 or Th2 pathway. It is important to note that these compounds specifically induce or release cytokines in dependence on antigen properties. They are thus distinguished from adjuvants which, in accordance with the present invention, unspecifically and broadly support an immuno response.

Application of the vaccine of the present invention allows, in conclusion, therefore the fine tuning of a desired immuno response to a given antigen, the nature of which also plays a given role, of course. This immuno response may be enhanced by an unspecific immuno response, triggered, for example, by an adjuvant. The option of fine tuning the immuno response is in particular advantageous over the prior art using only the injection of vaccines because the injection process per se will heavily and unspecifically disturb the relative cytokine concentrations in the skin.

Consideration of the above mentioned criteria not only provides the basis for a suitable kind of immunopresentation to the cells of the skin and peripheral immune system (by the carriers) but also ensures such immunoprocessing that will either predominantly generate antigen-neutralizing antibodies in the body, will give rise to the cell-mediated

immune response, or else will result in the gradual development of tolerance against the antigens or in the specific promotion of cell-mediated immunity.

In accordance with the present invention, it was also found that the outcome of noninvasive transcutaneous vaccination is strongly affected by the immuno-penetrant (antigen carrier) composition. Using antigens of different purity, unexpectedly, resulted in vastly different immune response. This was reflected in the observation that organisms with a similar overall titre revealed diverse levels of protection, probably due to the different final antigen isotype patterns.

Furthermore, the addition of a conventional, low molecular weight immunoadjuvant, monophosphoryl lipid A, not only made the result of epicutaneous immunization more robust, as documented by the smaller standard deviation in the measured antibody titres published before. Using this immunoadjuvant in immuno-carriers also, unexpectedly and contrary to previous experience obtained in mice, increased the secretion of IgG2b, and less strongly of IgG2a, but did not enhance IgA production. As the presence of IgG1, which is a Th1-like immunoglobulin, is inferred to be essential for, at least murine, protection against the tetanus toxin, the role of lipid A or bacterial antigens was thus revealed for the first time. For the future medical and commercial use of teachings disclosed in this invention it is important to realize that a high (specific) antibody titre does not necessarily imply a good protection result; to achieve the desired and sufficient protection the right kind and relative amount of certain antibody isotypes is required, such that will give prevalently Th1- or Th2-type of immune response (see previous discussion), as the case should be.

Basic formulations suitable for achieving the desired goals are known in the art; see, e.g., DE 41 07 152, PCT/EP91/01596, PCT/EP96/04526, DE 44 47 287, for more detailed or complementary information. The vaccine of this invention is not useful just for prophylactic or therapeutic vaccination but, moreover, is applicable for the treatment of allergy and for obtaining immunity against microbes, including extracellular and intercellular bacteria, viruses and parasites in the human and veterinary medicine.

In combination with the above mentioned penetrants, an antigen, such as an immunoactive substance, is transported across the barrier in form of a physical or a chemical complex with the former.

In order to profit from the pool of cytokines residing in the skin, a particularly useful method of vaccination is proposed in which an immunogen is applied on the skin after pre-treating the organ by an immunoadjuvant manipulation as defined before.

It is particularly advantageous to use the readings from the above mentioned local immune response to a patch assessment for optimizing the details and the course of further allergen administration, and thus to positively affect the outcome of therapeutic or prophylactic vaccination. It is believed that such an approach could be used advantageously to reach or improve immuno-tolerance of the tested subject to an applied allergen.

If primary immunization is done invasively, typically by using a subcutaneous injection or some other suitable skin barrier perforating/destroying method, one expects to obtain high IgM levels but the subsequent, booster immunizations may then be done non-invasively as described in this invention.

Finally, several optimization methods are proposed which can be used to improve immunogens and vaccination based on highly deformable penetrants. Preferred is a method wherein the flux of penetrants associated with an immunogen through the various pores in a well-defined barrier is determined as a function of suitable driving force or pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimize the formulation or application further. Its core is the determination of the flux of immuno-penetrants through the pores in a well-defined barrier as a function of suitable driving force or pressure, which acts across the barrier, and the resulting data analysis in terms of a characteristic curve which, in turn, can be employed to optimize the formulation or application further, based on comparison of different data sets. This includes comparison with the results pertaining to the immunogen-free penetrant suspensions of known skin penetration capability, reported for example by Cevc et al., (1998, *op. cit.*). In a complementary, preferred, embodiment various combinations of

immunomodulants or of immunomodulating procedures are tested with regard to chiefly Th1- or Th2-related cytokine production and the results are then used to make a suitable choice for the final therapeutic or prophylactic application.

Vaccination is typically done at ambient temperature, but lower or higher temperatures may also be suitable. They make particular sense with the formulations comprising synthetic substances which are rigid between the room and the skin or other barrier temperature.

Manufacturing temperature is normally chosen in the 0 to 95°C range. Preferably, one works in the temperature range 10-70°C, most frequently at temperatures between 15°C and 45°C, under all circumstances below the temperature at which any important formulation ingredient would undergo an irreversible change in composition or physical state. The skin temperature is normally 32°C. Other temperature ranges are possible, however, most notably for the systems containing freezable or non-volatile components, cryo- or heat-stabilized formulations, etc.

If required to maintain the integrity and the desired properties of individual system components, carrier formulations can be stored in cold (e.g. at 4°C), with or without an associated antigen. Manufacturing and storage under an inert atmosphere, e.g. under nitrogen, is possible and sometimes sensible. The shelf-life of immunogen formulation can also be extended by using substances with only a small number of double bonds, that is, by a low degree of unsaturation, by the addition of antioxidants, chelators, and other stabilizing agents, or by preparing the immuno-penetrants *ad hoc* or *in situ* from a freeze dried or a dry mixture.

In a preferred embodiment of the vaccine according to the invention the compound which specifically releases or specifically induces molecules with cytokine or anti-cytokine activity and the antigen are associated with the penetrant.

In a further preferred embodiment of the vaccine according to the present invention the less soluble self-aggregating molecule is a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of polar lipid which is sufficiently soluble for the purpose of this invention.

In an additional preferred embodiment of the vaccine according to the present invention the average diameter of the penetrant is between 30 nm and 500 nm, preferably between 40 nm and 250 nm, even more preferably between 50 nm and 200 nm and particularly preferably between 60 nm and 150 nm.

The invention in one further preferred embodiment relates to a vaccine wherein total weight of droplets in the formulation for the use on human or animal skin is 0.01 weight-% (w-%) to 40 w-% of total mass, in particular between 0.1 w-% and 30 w-%, and most preferably between 5 w-% and 20 w-%.

In another preferred embodiment of the vaccine according to the present invention total antigen concentration is between 0.001 w-% and 40 w-% of the total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 20 w-% and most preferably between 0.5 w-% and 10 w-%.

In another preferred embodiment of the vaccine according to the present invention the formulation further comprises (da) a low molecular weight chemical irritant, and/or (db) a low molecular weight compound from a pathogen or a fragment or a derivative thereof.

In yet another preferred embodiment of the vaccine according to the present invention the compound exerting cytokine activity is IL-4, IL-3, IL-2, TGF, IL-6, IL-7, TNF, IL-1 α and/or IL-1 β , a type I interferon, preferably IFN- α or IFN- β , IL-12, IFN- γ , TNF- β , IL-5 or IL-10.

In one more preferred embodiment of the vaccine according to the present invention the compound displaying anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative or an analogue thereof.

The term "active fragment or derivative thereof" in this connection means that the above-recited activity is essentially maintained or mimicked by the substance used.

In another preferred embodiment of the vaccine according to the present invention the antigen is derived from a pathogen.

In another particularly preferred embodiment of the vaccine according to the present invention said pathogen is selected from extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus*, gram-negative bacteria, such as *Meningococcus* and *Gonococcus* species, species of *Neisseria*, gram negative bacteria, including enteric organisms such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Diphtheria*, *Bordetella Pertussis*, and gram-positive bacteria (e.g. *Bacillus pestis*, BCG), particularly anaerobes, such as the *Clostridium* species (e.g. *Clostridium tetani*, *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*), bacteria and viruses, which survive and replicate within host cells, comprising mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes*, retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species (e.g. *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae*, *B. ovis*), the causative agent for cholera (e.g. *Vibrio cholerae*), *Haemophilus* species like *H. actinomycetemcomitans*, *H. pleuropneumoniae*, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases and pathogens that cause various neoplasiae, auto-immune diseases or are related to other pathological states of the animal or human body which do not necessarily result from pathogen infections.

In a preferred embodiment of the vaccine according to the present invention the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant which leads to an acute hypersensitivity reaction of the body exposed to the allergen, many such allergens stemming from mite, pollen, animal hair or skin debris, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.

In a further preferred embodiment of the vaccine according to the present invention the concentration of each compound displaying cytokine activity used is selected to be up to 1000 times higher than the concentration optimum established in the corresponding tests with the antigen and immunoadjuvant chosen, performed by subcutaneously injecting the formulation or performing the tests *in vitro*, and preferably is up to 100x, more often up to 50x and even better up to 20x higher.

In a different preferred embodiment of the vaccine according to the present invention the pathogen extract or compound is a lipopolysaccharide, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a pathogen; an extract of a pathogen, including bacterial exo- and endotoxins, preferably cholera toxin or the heat labile toxin (HLT) of *E. coli*, an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, or a purified protein derivative of *M. tuberculosis*, LT-R192G, Fibronectin-binding protein I of *Streptococcus pyogenes*, or outer membrane protein of group B *Neisseria meningitidis* (GBOMP); or bacterial or viral nucleic acids such as oligonucleotides containing unmethylated CpG dinucleotides.

In a particularly preferred embodiment of the present invention said lipopolysaccharide is lipid A or a derivative and modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose.

In another particularly preferred embodiment of the vaccine according to the present invention the concentration of the pathogen compound derived from a pathogen is between 10x lower and up to 1000x higher than that otherwise used with the corresponding injected formulations employing similar antigen, the epicutaneously administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.

In still another particularly preferred embodiment of the vaccine according to the present invention the low molecular weight irritant is selected from the classes of allergenic metal ions, acids, bases, irritating fluids, (fatty-) alcohols, (fatty-) amines,

(fatty-) ethers, (fatty-) sulphonates, -phosphates, etc., or other suitable solvents or amphiphiles, or from the group of surfactant-like molecules, often with the skin permeation enhancing capability, as well as derivatives or combinations thereof.

In a preferred embodiment of the vaccine according to present invention the concentration of a low molecular weight irritant is chosen to be by at least the factor of 2, more often by the factor of 5, and even better by the factor of 10 or more, below the concentration which in independent tests on the same or comparable subject is deemed to be unacceptable owing to the local irritation, as assessed by the methods and standards commonly used to test such an irritant.

In a further particularly preferred embodiment of the vaccine according to the present invention the allergen belongs to the class of inhalation allergens, including various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; food and drug allergens; contact allergens; injection, invasion and depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., parts of implantation material, etc..

In a preferred embodiment of the vaccine according to the present invention the applied dose of an antigen differs by the factor of 0.1 to 100 from the dose which otherwise would have to be injected in the process of immunization, but more often is in the range between 0.5 to 50, even better between 1 and 20 and ideally is less than 10x higher than that used with an injection.

In another preferred embodiment of the vaccine according to the present invention the applied penetrant dose is between 0.1 mg cm^{-2} and 15 mg cm^{-2} , even more often is in the range 0.5 mg cm^{-2} and 10 mg cm^{-2} , and preferably is between 1 mg cm^{-2} and 5 mg cm^{-2} . It may also be advantageous to use different administration areas to control the applied immunogen dose, using easily accessible or sheltered body areas (such as the chest or back regions, arms, lateral side of the neck, e.g. behind the ears, or even in the scalp region) for the purpose.

In a different preferred embodiment of the vaccine according to the present invention said antigen is a pure or purified antigen. The use of highly purified antigens in the

vaccine of the invention has turned out to be particularly advantageous for the generation of a protective immuno response.

The present invention further relates to a kit comprising, in a bottled or otherwise packaged form, at least one dose of the vaccine.

In a preferred embodiment according to the present invention the kit comprises at least one injectable dose of the antigen described above.

The present invention further relates to a method for generating a protective immune response on a mammal comprising vaccinating said mammal with a vaccine as described above.

In another preferred embodiment of the method according to the present invention different treatment areas are selected to control the applied immunogen dose and the outcome of therapeutic vaccination.

In one more preferred embodiment of the method according to the present invention a suspension of antigen-free penetrants is loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the administration of resulting formulation on the skin.

In a different preferred embodiment of the method according to the present invention the vaccine of the present invention is applied on the skin after pre-treating the organ by an immunoadjuvant manipulation, said manipulation comprising, for example, skin rubbing, pressing, heating, exposing to an electrical or mechanical, e.g. ultrasound field, etc., or injecting a non-immunogenic formulation in the skin, provided that any such treatment releases immunoadjuvant compounds from the skin or other peripheral immuno-active tissues or else reduces the concentration/duration of action of antagonists to the desired vaccination.

In a preferred embodiment of the method according to the present invention immunogen is applied in a non-occlusive patch. This embodiment can also be used for

the purpose of assessing the skin reaction to an epicutaneously administered immunogen in the penetrant suspension, to which the former, at least originally, is allergic and which thus gives rise to an acute local hypersensitivity reaction, as seen, for example from the resulting flare, irritation, etc.

In another preferred embodiment of the method according to the present invention at least one dose of vaccine is administered.

This embodiment of the method of the invention includes the repeated administration of the vaccine of the invention. Repeated administration includes repeated administration on the skin or one or more administrations on the skin in combination with, e.g., parenteral administrations. In this connection, the kit of the invention may be advantageously used that comprises one or more containers or ampoules comprising the vaccine of the invention.

In a particularly preferred embodiment of the method according to the present invention said vaccine is administered as a booster vaccination.

In a most preferred embodiment of the method according to the present invention the primary immunization is done invasively, typically using a subcutaneous injection or some other suitable skin barrier perforating/destroying method, and the at least one subsequent, booster immunization is done non-invasively.

In a preferred embodiment of the method according to the present invention the vaccine is applied between 2 and 10, preferably between 2 and 7, even more preferably up to 5 and most preferably up to 3 times, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined according to a suitable assessment method, or else to deem the effort as having failed.

In a particularly preferred embodiment of the method according to the present invention the time interval between the subsequent vaccinations is chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years. In a further preferred embodiment,

repeated immunogen administration is advocated to maximize the final effect of a therapeutic vaccination. It is proposed to use between 2 and 10, often between 2 and 7, more typically up to 5 and most preferred up to 3 immunizations, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined as described above or another suitable assessment method, or else to deem the effort as having failed. The time interval between subsequent vaccinations should preferably be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years, when a subject is being immunized for the first time. Rodents, such as mice and rabbits are advantageously immunized in 2 weeks interval, primates, e.g., monkeys and often humans, need a booster vaccination in 3-6 months interval.

In a preferred embodiment of the method according to the present invention the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is determined as a function of a suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimize the formulation or application further.

The invention finally relates to the use of the transdermal carrier, the compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity, the antigen or allergen, and optionally an extract or a compound from a microorganism or a fragment or a derivative thereof, and/or a low molecular weight chemical irritant as defined hereinbefore for the preparation of a vaccine for inducing a protective or tolerogenic immune response.

The figures show:

Figure 1 gives the data on survival of animals immunized epicutaneously with mixed micelles or Transfersomes loaded with TT, to illustrate aggregate size (stability) effect, since the over-destabilized Transfersomes normally disintegrate into the mixed lipid micelles.

In figure 2 the comparison is made between the immune response to conventional lipid vesicles (liposomes) and ultradeformable lipid vesicles (Transfersomes) carrying TT and applied on the skin, the information on corresponding specific antibody concentrations in serum (expressed as absorbance) being given in upper panel.

Figure 3 illustrates the effect of increasing antigen dose on the outcome of epicutaneous immunization by means of Transfersomes, the results being expressed as absorbance change, antibody titre, or animal survival, together with the corresponding specific antibody isotyping data.

Figure 4 highlights the effect of antigen purity on the result of epicutaneous immunization with tetanus toxoid in Transfersomes, including information on time dependence of animal survival.

Figure 5 compares the outcome of repeated invasive (subcutaneous) and non-invasive (epicutaneous) immunization by means of TT in Transfersomes, including animal survival, serum concentration (in terms of absorbance), specific antibody titre, and antibody distribution pattern values.

Figure 6 illustrates the effect of skin pre-treatment (non-specific challenge) on the immune response following Transfersome mediated TT delivery across the skin.

Figure 7 focuses on adjuvant effect of a relatively low-molecular weight immuno-stimulator, monophosphoryl Lipid A (LA), delivered across intact skin together with TT in Transfersomes.

Figure 8 demonstrates the immuno-adjuvancy of a cytokine, interleukin-12 (IL-12) transported across the skin together with TT by means of Transfersomes.

Figure 9 deals with the immuno-modulation by various cytokines of the murine response against TT antigen delivered in Transfersomes non-invasively through the skin.

Figure 10 presents experimental evidence for the immune response stimulation of mice treated on the skin by TT in Transfersomes, when the carriers also include cholera toxin (CT) to support the specific antibody production, and thus animal protection against an otherwise lethal challenge by the tetanus toxin.

Figure 11 illustrates the use of heat labile toxin from *E. coli* as an immuno-adjuvant.

Figure 12 illustrates the immuno-modulating effect of local skin pre-treatment with histamine in combination with transdermal antigen application with Transfersomes.

Figure 13 demonstrates the effect of subcutaneous priming on anti-tetanus titer and on the survival of epicutaneously vaccinated hosts.

Figure 14 show the effect of bi-valent vaccination with Tetanus Toxoid and Cholera Toxin used as antigens.

The examples illustrate but do not define the limits of the invention.

General experimental set-up and sample preparation

Mice of Swiss albino strain (18-20 g) were obtained from The National Institute of Nutrition (Hyderabad, India). They were 8 to 12 weeks old at the time of first immunization and were normally kept in suspension cages in groups of 4 to 6. The animals had free access to standard chow and water. One day prior to an immunization, the application area on murine back was shaved carefully. The antigen was administered with a high precision pipette on the skin surface and left to dry out partially. To prevent immunogen abrasion, the animals were transferred into individual cages in which they were kept for 18 hours following each epicutaneous material administration.

General anesthesia was used to keep the test animals stress free and quiet during manipulations, including immunization. An injection of a mixture of Ketavet and Rompun (0.3 mL per mouse of an isotonic NaCl solution containing 0.0071 % Rompun

(Bayer, Leverkusen, Germany) and 14.3 mg/mL Ketavet (Parke-Davis, Rochester, N.Y) into the peritoneal cavity was used for the purpose. This typically kept the animals asleep for app. 2 hours.

Immunogens

Ultradeformable immuno-carriers, or immuno-penetrants (immuno-Transfersomes), studied in this work, typically had the form of (oligo)bilayer vesicles. They contained biocompatible (phospho)lipids, such as phosphatidylcholine, and (bio)surfactants, such as sodium cholate or polysorbate (Tween 80), different compositions maintaining the high aggregate deformability being possible. Additional ingredients were monophosphoryl lipid A, with a versatile immunoadjuvant activity, and antigens, as required and specified.

Conventional vesicles, liposomes, comprised soy phosphatidylcholine (SPC; Nattermann Phospholipids, Rhone-Poulenc Rorer, Cologne, Germany) and were prepared as described as follows. An organic lipid solution with or without the adjuvant monophosphoryl lipid A (MLA) at 0.04 mol-% relative to SPC was first dried under vacuum (10 Pa, overnight). The resulting lipid film was hydrated with a solution of tetanus toxoid (2.0 mg/mL; Accurate antibodies, NY, USA) in phosphate buffer (pH = 6.5) to get a 10 wt-% lipid suspension. Crude suspension of lipid vesicles was extruded through the series of polycarbonate membranes with 800 nm, 400 nm, and 200 nm pores, to narrow down the final vesicle size distribution.

Highly deformable vesicles, Transfersomes, were prepared as described earlier (Paul et al., 1995 *op. cit.*). In short, an ethanolic SPC solution was mixed with sodium cholate (Merck, Darmstadt, Germany) (3.75/1 mol/mol) and the adjuvant, if required. The mixture was dispersed in 10 mM phosphate buffer (pH = 6.5). This was done with tetanus toxoid present in the solution to give between 0.25 mg and 2.0 mg protein per 1 mL of suspension, as required. Vesicle suspension was then frozen and thawed three times. Subsequently, the formulation was passed through a micro-porous filter (200 nm; Poretics, CA) under pressure. To check the reproducibility of vesicle manufacturing, the optical density at 400 nm was measured with each preparation and confirmed to be approximately constant.

By varying surfactant-to-lipid ratio the vesicular aggregate deformability was controlled, up to the concentration at which membranes became unstable, owing to the high surfactant concentration, and reverted into a micellar form. Lipid vesicles without the surfactant added, which are commonly known as liposomes and have at least 10x less flexible membranes than Transfersomes, were used as negative controls.

Total lipid concentration was typically 10 w-%, unless stated otherwise. Antigen concentration was typically, but not necessarily, of the order of 1 mg/mL. A buffer containing microbicide provided the bulk phase. For other suitable compositions the expert is explicitly referred to other publications and patents from our laboratory.

Immunizations were done with different formulations, including the ultradeformable vesicles without antigens; such vesicles then contained the tetanus toxoid (with or without lipid A) and free immunogen. Each formulation was tested on six mice, unless stated otherwise.

In the case of subcutaneous immunization, 40 μ g of immunogen was injected per mouse. For a non-invasive administration, tetanus toxoid doses between 1 μ g and 80 μ g, associated with different carriers, were administered per mouse on the intact skin of upper dorsum. All non-injected formulations were applied with a high precision pipette and left to dry; during this period mice were kept in separate cages to minimize the applied material abrasion, such as might result from the rubbing of the murine backs on each other. Animals were boosted every two weeks, that is on days 14 and 28; the total immunization scheme thus consisted of three doses, and comprised a prime and two boosts.

Animals were bled retro-orbitally on the days 7, 21 and 35. The collected blood was first allowed to clot. After a brief centrifugation in a micro-centrifuge the serum was separated, de-complemented at 56 °C for 30 min, and then stored at -20 °C, until the total antibody concentration and the specific antibody isotypes was determined.

Absorbency measurements were done using standard UV-vis spectrometer.

Measurement of tetanus toxoid (TT) specific antibodies in serum by ELISA. The level of anti-tetanus antibodies was determined by ELISA in the customary fashion, typically in a duplicate. In brief, ELISA plates (maxisorp: NUNC, Germany) were coated with an aliquot (100 μ L containing 10 μ g of TT/mL) in coating buffer ($\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH=9.6) for 3 hours at 37°C. Wells were first washed thrice with 200 μ L/well of washing buffer and then blocked with 2% milk in washing fluid (for 1000 mL, 8 g NaCl, 1.45 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 g KH_2PO_4 , 0.2 g KCl and 0.05% Tween-20) for 3 hours at 37°C. After single wash with 200 mL/well of washing buffer, the plates were incubated with various dilutions (1/50 to 1/6400) of the test serum. After an overnight incubation at 4°C the plates were washed thrice with 200 μ L/well of washing buffer and incubated with 100 μ L of secondary antibody. When determining the amounts of IgG, IgA, or IgM, horse radish peroxidase (hrp) conjugated to the appropriate Anti-Ig was used. After a 3 hours incubation at 37°C, the plates were washed thrice with 200 μ L/well of washing buffer and the color was developed using o-phenyl diamine as hrp substrate. 0.4 mg/mL of o-phenyl-diamine in phosphate-citrate buffer (pH 4.5) with 0.4 μ L H_2O_2 per mL was used for the purpose. After 2 minutes the reaction was stopped by the addition of 50 μ L of 2N H_2SO_4 . The absorbency was measured at 492 nm.

The method used to detect various isotypes was also ELISA based. It relied on the peroxidase-labeled, affinity purified secondary antibodies specific for IgG1 (1:1000), IgG2a (1:1000), IgG2b (1:1000), and IgG3 (1:200) which were all obtained from ICN ImmunoBiologicals. Further secondary antibodies included IgA (1:1000) and IgM (1:1000) linked to horse-radish peroxidase (Sigma, Neu-Ulm, Germany). The correspondingly labeled anti-mouse IgE was purchased from PharMingen (San Diego, CA). The antigens were again permitted to adsorb on test plates and incubated with the test serum after excess of the antigen had been washed away. Subsequently, 100 μ L of appropriate specific secondary antibody solution was added to one of the six different plates, to determine anti-IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, respectively. The plates were incubated for 3 hours at 37°C and processed further as described in previous paragraphs.

Challenge with antigen (the tetanus toxin) in vivo. On the day 35, test animals

were challenged by injecting 50 times the LD₅₀ of the tetanus toxin subcutaneously (s.c.). (The actual value of LD₅₀ was fixed in separate experiments, during which a group of 16 weight-matched animals was challenged s.c. with increasing amounts of toxin and the number of survivors was determined.) To determine the acute TT toxicity in vaccinated animals, the clinical status of such test mice was recorded for 4 days after the first challenge.

Non-protected mice showed signs of paralysis after 24 hours resulting in death, after 36 hours, at latest. Animals which developed no symptoms of paralysis or other anomaly over a 4 days period following the challenge were deemed immune against tetanus.

The long-term immunity was tested by challenging all immunized mice on a monthly basis with a dose of toxin corresponding to 50 times LD₅₀, for at least half a year.

Examples 1-2:

Aggregate size (stability) effect

Highly deformable vesicles (Transfersomes™: IDEA):

- 87.4 mg phosphatidylcholine from soy bean (SPC)
- 12.6 mg sodium cholate (NaChol)
- 0.04 mol-% monophosphoryl Lipid A (MLA, LA) relative to SPC
- 0.9 mL phosphate buffer, 10 mM, pH 6.5
- 0.1 mL ethanol

(Mixed lipid) Micelles:

- 65 mg phosphatidylcholine from soy bean (SPC)
- 35 mg sodium cholate (NaChol)
- 0.04 mol-% monophosphoryl Lipid A (MLA) relative to SPC
- 0.9 mL phosphate buffer, 10 mM, pH 6.5
- 0.1 mL ethanol

Tetanus toxoid (2 mg/mL; Accurate Antibodies) used at the dose of
40 µg (20 µL) or 80 µg (40 µL) TT per mouse and immunization

Application area: 1 cm² or 2 cm² for 40 µg or 80 µg TT per mouse on the upper dorsum.

To test the effect of formulation stability on the immunological properties of various, epicutaneously administered formulations, two kind of aggregates were prepared: relatively large vesicles (diameter between 100 nm and 200 nm) and relatively small micelles (diameter below 50 nm). The latter were chosen in the expectation that under suboptimal conditions (owing to the lipid degradation or inappropriate aggregate composition) the latter may arise from the former.

Antibody titres, as reflected in the serum absorbency at 492 nm, are shown in figure 1. They show that mixed lipid micelles are less efficient antigen carriers than ultradeformable mixed lipid vesicles (Tfs) loaded with the same amount of TT. Mixed micelles containing less potent detergents (with lesser skin permeation enhancing capability) were even less efficient immune response mediators.

Animal protection data reveal a similar trend, as is seen in lower panel of figure 1.

Examples 3-4:

Aggregate deformability effect

Conventional lipid vesicles (liposomes):

- 100 mg phosphatidylcholine from soy bean (SPC)
- 0.4 mol-% monophosphoryl Lipid A (MLA) relative to SPC
- 0.5mL phosphate buffer, 10 mM, pH 6.5
- 2 mg/mL tetanus toxoid (Accurate Antibodies)

Highly deformable vesicles (Transfersomes™):

- 87.4 mg phosphatidylcholine from soy bean (SPC)
- 12.6 mg sodium cholate (NaChol)
- 0.04 mol-% monophosphoryl Lipid A (MLA) relative to SPC
- 0.9 mL phosphate buffer, 10 mM, pH 6.5
- 0.1 mL ethanol

Tetanus toxoid used at the dose of 40 µg or 80 µg TT/ mouse/ immunization

Application area: 1 cm² or 2 cm² for 40 µg or 80 µg TT/ mouse/ immunization on the upper dorsum.

Results obtained with the conventional vesicles differ from the data measured with highly deformable vesicles: simple liposomes, which do not cross the narrow pores in a barrier also do not elicit a substantial antibody titre. Conversely, the vesicles with a highly flexible and deformable, and thus better adaptable, membrane which were shown separately to move through the narrow pores in a barrier with greater ease, generate an appreciable quantity of antibody when applied on intact skin, according to the results of serum absorbency measurements (cf. figure 2).

Examples 5-10:

Antigen dose effect

Highly deformable vesicles:

- 86.3 mg phosphatidylcholine from soy bean (SPC)
- 13.7 mg sodium cholate (NaChol)
- 0.04 mol-% monophosphoryl Lipid A (MLA) relative to SPC
- 0.9 mL phosphate buffer, 10 mM, pH 6.5
- 0.1 mL ethanol

Tetanus toxoid (TT: Accurate Antibodies, New York, USA) concentration:

- empty, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, giving rise to
- 0 µg, 10 µg, 20 µg, 40 µg or 80 µg TT/ mouse/ immunization

Application area: 1 cm² for 0 µg, 10 µg, 20 µg, 40 µg and 2 cm² for 80 µg TT/mouse/immunization on the upper dorsum.

The results of this experimental series are illustrated in figure 3. It clearly shows the increase in immune response to epicutaneously administered tetanus toxoid in ultradeformable carriers with increasing TT dose. This is reflected in serum absorbency (up to the dose of 20 µg/immunization), in specific antibody titre (up to the dose of 40 µg/immunization), and in the survival data (which do not saturate for doses up to 80 µg/immunization).

Less clarity is found in isotype distribution pattern, except for IgG1 (with a strong indication for the response saturation) and for IgG2b (perhaps, with the saturation between 40 μ g and 80 μ g per immunization). IgM shows dose dependence similar to that of IgG1. The picture obtained for IgG2a is confusing.

Examples 11-13:

Antigen purity effect

Highly deformable vesicles:

as described with examples 5-10 (except in that the group treated with impure TT did not receive immunoadjuvant lipid A)

Tetanus toxoid: 2 mg/mL, corresponding to 80 μ g TT per mouse/ immunization

Application area: 2 cm² on the upper dorsum.

Antigen purity strongly affects the level of murine protection against tetanus toxin when the toxoid has been applied non-invasively on the skin. (Similar results obtained with injected antigen are not shown).

To substantiate the above mentioned statement, the medium filtrate from a culture of *Clostridium tetani* grown in vitro first was used as an impure antigen. To obtain partially purified antigen, such filtrate was passed through a 10 kDa cut-off membrane and washed thoroughly with phosphate buffer, pH 6.5; in the process, the culture filtrate was concentrated 15 times. Purified toxoid was purchased from Accurate Antibodies, NY, USA.

Swiss albino mice (n = 6) were immunized with identical nominal dose of impure antigen, with partially purified antigen supplemented with monophosphoryl lipid A or with purified antigen with monophosphoryl lipid A added. The antigen was always associated with similar Transfersomes. The composition and the method of preparation for the latter were the same as described with previous examples. The details of immunization schedule, bleeding times and challenge as well as analysis details were also similar to those mentioned before.

The results are given in figure 4. They demonstrate the role antigen purity plays in determining the quality as well as the strength of an immune response against TT. The data shown in figure 4, moreover, indicate that the absorbency of even the specific antibody titre is not a reliable predictor of the therapeutic, that is, of prophylactic effect of an epicutaneous vaccination. This is due to the big differences in specific antibody isotypes which only contain a substantial proportion of Th1-like IgG2b compared to Th2-like IgG1 component if sufficiently pure antigen is used (see also page 12).

Examples 14-15:

Comparison of epicutaneous and subcutaneous administration

Highly deformable vesicles, Transfersomes™ (IDEA):

as described with examples 5-10

Tetanus toxoid dose:

80 µg TT per epicutaneous immunization

(using 2 mg TT/mL and application area of 2 cm²)

40 µg TT per subcutaneous injection (using 2 mg TT/mL)

Using the same experimental procedures as described with examples 1-4, as appropriate, the antibody-specific serum titre, the level of animal protection against tetanus toxin and relative occurrence of different specific antibody isotypes was determined.

The results are given in figure 5. While the immunization dependent increase in serum absorbency is comparable after invasive and non-invasive antigen administration the titre in the latter case is somewhat lower by the factor of 6 after primary immunization and by the factor of 8 after second boost. Likewise, while the TT-specific levels of Th2-indicating IgG1 are similar in both arms of this experiment, the specific readings for other antibody subtypes, especially for IgG2a and at early time points also for IgG2b are by the factor 25 and 3, respectively, higher after antigen injections. However, the likelihood for the test mice to survive a subsequent challenge with a normally lethal dose of injected tetanus toxin is independent of the route of antigen administration, within the framework of this experimental series at least.

Examples 16-17:Adjuvant skin treatment (pre-injection) effect

Highly deformable vesicles, Transfersomes™ (IDEA):

89.3 mg phosphatidylcholine from soy bean (SPC)

10.7 mg sodium cholate (NaChol)

0.04 mol-% monophosphoryl Lipid A (MLA) relative to SPC

0.9 mL phosphate buffer, 10 mM, pH 6.5

0.1 mL ethanol

Tetanus toxoid, 2 mg/mL, corresponding to 80 µg TT per mouse/ immunization
of 6 Swiss albino mice per group using impure antigen

Application area: 2 cm² on the upper dorsum.

Transcutaneous transport of macromolecules associated with Transfersomes across the skin seems to be extremely gentle; it therefore fails to trigger the immune system toward Th2-like immune response, if the antigen is used in a low amount or is impure. To change the situation, the skin can be (pre)stimulated to release corresponding messenger molecules from the organ prior to the actual non-invasive antigen administration by means of ultradeformable vesicles. For this purpose we have pre-injected the application site with 0.1 mL of saline, or a mild formulation of non-antigenic vesicles, prepared from biodegradable material of similar composition as the antigen carrying vesicles one day before using the latter. For additional control, incomplete Freund's adjuvant was also injected in different animals 24 hours before the application of immuno-carriers on the skin.

Illustrative examples of results are given in figure 6. They reveal higher specific antibody titres, especially for and improved protection in the mice that were pre-treated by injections rather than carrier formulation on the skin, which served as a control. The effect of incomplete Freund's adjuvant is surprisingly weak.

It is noteworthy that the serum absorbency or the specific antibody titre and animal survival, that is protective vaccination effect, are not correlated.

Examples 18-21:Low molecular weight adjuvant (lipid A) effect

Highly deformable immuno-modulated TT-Transfersomes™ (IDEA):

- 86.3 mg phosphatidylcholine from soy bean (SPC)
- 13.7 mg sodium cholate (NaChol)
- 0.04 mol-% monophosphoryl Lipid A (LA) relative to SPC
- 0.9 mL phosphate buffer, 10 mM, pH 6.5
- 0.1 mL ethanol

Highly deformable standard immuno-vesicles, TT-Transfersomes™ (IDEA):

as above but without LA added

Tetanus toxoid: 2 mg/mL, with 20 μ L or 40 μ L corresponding to 40 μ g or 80 μ g TT per immunization

Application area: 1 cm² or 2 cm², respectively, on the upper dorsum.

We believe that immuno-active, typically immunopotentiating, molecules must be present in the skin at the time of antigen presentation to the body by TT carriers that have crossed the barrier in order to achieve the desired immunological action of the antigen. To substantiate this conclusion we compared the outcome of non-invasive immunopresentation of TT by means of Transfersomes with or without a well known immunostimulant, monophosphoryl lipid A (LA), which is known to elicit generation of TNF in the body, for example. Two different antigen doses were used. In either case substantial titres and a measurable prophylactic immune response (partial immunity) was reached.

The absorbency of the serum increases as one would expect (cf. figure 7). Conversely, the effect of LA is better seen for the lower than for the higher dose used. This may be due to experimental variability or else reflect non-linearity of dose vs. action curve for the typical immunization data. It is possible, for example, that adjuvant is only efficient in the low dose range, whereas in the high dose regiment the system is quasi-saturated, leaving little possibility for the adjuvant to further enhance the immune

response within the scope of experimental set-up. Complete animal protection against a normally lethal challenge with 50 LD₅₀ was achieved in this test series with the higher TT dose in combination with LA only.

It was further observed that Th1-cytokine IgG2b was higher with LA groups, compared with the groups that received no LA. This difference was more pronounced for low doses, by the factor of 4, than for high doses, where only an enhancement by the factor of 2 was observed. Th2-cytokine IgG1 was present predominantly, except in the low dose with LA group in which IgG2b contributed comparably.

Examples 22-23:

High molecular weight adjuvant, IL-12 cytokine effect

Highly deformable vesicles, Transfersomes™ (IDEA):

as described with examples 5-10, plus

0.01 mg IL-12 per mL immunogen suspension

Tetanus toxoid, 2 mg/mL, corresponding to 80 µg TT per mouse/ immunization
(partially purified as described with examples 9-11)

Application area: 2 cm² on the upper dorsum of Swiss albino mice.

To study the effect of cytokines on results of non-invasive, epicutaneous vaccination with tetanus toxoid, a combination of monophosphoryl lipid A with 0.4 µg IL-12 per mouse was used. 80 µg of IL-12 was administered per mouse in association with Transfersomes loaded with tetanus toxoid and monophosphoryl lipid A. The details of immunization schedule, bleeding intervals, or the final challenge with the tetanus toxin were the same as mentioned above.

The results of experimental series are illustrated in figure 8. The corroborate the conclusion that the presence of pro Th2 cytokines in the skin during the course of immunopresentation following an epicutaneous TT administration positively affects the outcome of vaccination. This is seen in serum absorbency, the specific antibody titre as well as in the test animal survival probability.

The effect discussed with examples 22-23 was verified by incorporating cytokines other than IL-12 into immunogen formulation. The results are shown in figure 9.

Examples 24-25:High molecular weight adjuvant (IFN- γ and GM-CSF + IL-4) effect

Highly deformable vesicles, Transfersomes™ (IDEA):

as described with examples 5-8, plus

0.05 mg IFN- γ and 0.004 mg GM-CSF and 0.004 mg IL-4 per mL
immunogen suspension

Tetanus toxoid, 2 mg/mL, corresponding to 80 μ g TT per mouse/ immunization
(impure)

Application area: 2 cm² on the upper dorsum of Swiss albino mice.

The effect discussed with examples 22-23 was confirmed also with a blend of different cytokines. The results are shown in figure 10.

Examples 28-29:Booster effect (maturation of immune response)

In most of previous examples, a consistent pattern was observed whenever the absorbency was measured during the time course of immunization. The immune response increased with each boost, compared to the response obtained after primary immunization (see Figures 3, 4, 5, 6, 7, 8). The primary response was characterized by predominance of IgM, followed by gradual appearance of IgG after the first boost and by the appearance of even greater amounts of IgG after the second boost, with a concurrent disappearance of IgM. This typical pattern of isotype signifies affinity maturation in the immune response. During the process, the average affinity of a mixture of specific antibodies increases with repeated immunizations.

Results of various epicutaneous vaccination experiments suggest that it may be advantageous to combine an invasive priming vaccination with non-invasive secondary (boost) immunization.

Examples 30-72:Release of cytokines from the skin in vitro by Transfersomes

Highly deformable vesicles (Transfersomes type C):

87.4 mg phosphatidylcholine from soy bean (SPC)

12.6 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 50 mM, pH 7.3

2.5 μ L thereof

Highly deformable vesicles (Transfersomes type T):

50 mg phosphatidylcholine from soy bean (SPC)

50 mg polysorbate (Tween 80)

0.9 mL phosphate buffer, 50 mM, pH 6.5

2.5 μ L thereof

Positive control A:

2.5 μ L 5% sodium dodecylsulphate (SDS)

Positive control B:

100 μ L lipopolysaccharide (LPS; 10^5 U/mL)

Negative control:

2.5 μ L of phosphate buffered saline (PBS)

All products were tested undiluted.

Cell type: Normal human keratinocytes, forming a pluri-stratified epithelium with a compact stratum corneum were used; histology revealed strong resemblance with human epidermis in vivo.

Method: Keratinocytes were inoculated on polycarbonate filter inserts of 0.63 cm² in chemically defined, supplemented medium, and cultured for 17 days at the air-liquid interface.

Test measurements: given amount of each tested product was deposited with a micropipette and spread evenly over the surface of the stratum corneum of eight reconstituted epidermis using a small sterile device. The cultures were incubated at 37°C, 5% CO₂ for 24 hours. Quadruplicate cultures (except for the LPS treated cells which were incubated in duplicate) were washed with 0.5 mL of PBS and incubated on 300 μ L of 0.5 mg/mL MTT for 3 hours at 37°C, 5% CO₂.

The release of inflammatory mediators (IL1 α , IL2, IL4, IL8, IL10, IFN- γ , and TNF- α) in the medium underlying the tissues was quantified using ELISA kits (R&D systems UK; Quantikine), specific for each type of immuno-modulator to be measured.

	IL1- α (pg/mL) Mean +/- SD	IL8 (pg/mL) Mean +/- SD	TNF- α (pg/mL)
Negative control (PBS, n=2)	5.1 +/- 0.5	< 31	not detectable
Positive control A (SDS 5 %, n=2)	314.2 +/- 6.1	147.5 +/- 32	not detectable
Positive control B (LPS, n=1)	32.0	5161	113.4
Transfersomes C (02-05, n=2)	12.3 +/- 0.9	68.3 +/- 16.8	not detectable
Transfersomes T (TT0009/175, n=2)	11.7 +/- 1.2	50.8 +/- 14.0	not detectable
Transfersomes O (TT0017/15, n=2)	185.5 +/- 170.1	58.4 +/- 27.0	not detectable

The relatively big standard deviation observed with Transfersomes O can be explained by the fact that the product was difficult to spread uniformly onto the stratum corneum of the reconstructed epidermis.

TNF- α level was increased to the level of 113.43 pg/mL when the cells were in contact with the positive controls containing LPS, which is an established immunoadjuvant.

IL8 concentration after cells incubation with Transfersomes exceeded the lower limit of detection by just the factor of 2, which in one case is not and in the other is barely significant at 95% confidence level, but in either situation is negligible compared to the increase observed with the positive control containing the immunoadjuvant LPS, which gave a 167x higher value.

Non-specific irritant, SDS, released a great quantity of IL-1 α from the skin cells into the bathing medium in vitro. The possibility exists, that an amount of comparable quantity was released from the cells incubated with Transfersomes O, comprising the potentially irritating oleic acid at a high concentration, but firm conclusion is prevented by the great standard deviation in the results obtained with the latter test system.

IL-1 α concentration for the other tested Transfersomes of type A and type B changed to approximately 2 times the background level. This difference is statistically significant, compared to negative controls, but practically negligible, taken that the

increase observed with the positive control containing LPS was more than 60 times higher.

IFN- γ , IL-2, IL4 or IL10 was not elevated to a measurable level, suggesting a lack of release of these cytokines, under any other test condition.

Taken together the above mentioned findings suggest that Transfersomes do not release cytokines or induce the generation of such molecules from the skin cells. This explains the need for using immunoadjuvants/modulators when antigens or allergens are to be delivered across the skin with such carriers and elicit a therapeutic or prophylactic immune response.

Examples 73-82:

Bacterial wall component, cholera toxin, as specific immuno-adjuvant:

Highly deformable vesicles, Transfersomes™:

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

Cholera toxin (CT; Sigma, Neu-Ulm), 10 μ g/immunization plus, if required,

Tetanus toxoid (TT, pure; Accurate Antibodies) 2 mg/mL,

Volume doses corresponding to 0 μ g TT/mouse/immunization (negative control), 1 μ g TT/mouse, 5 μ g TT/mouse, 10 μ g TT/ mouse, 20 μ g TT/ mouse, 40 μ g TT/mouse (in the case of CT usage) and 80 μ g TT/mouse (without CT) were used epicutaneously over an area of up to 2 cm² on the upper dorsum of 4-6 Swiss albino mice; 20 μ g TT/ mouse/immunization were injected subcutaneously at the corresponding site in the positive control group. Unimmunized mice were used as another negative control.

The protective effect of epicutaneous antigen administration was excellent when cholera toxin was included into the test formulation in combination with the tetanus toxoid. Formulation without this immunoadjuvant yielded inferior protection, as demonstrated by the fact that 1 animal out of 4 (25%) was paralytic after the challenge with tetanus toxin.

The results shown in figure 10 reveal that the antigen doses in excess of 20 μg /immunization ensured complete protection, which was not the case with the other tested adjuvants or adjuvant treatments (see previous examples). Lower dosage of antigen gave qualitatively similar effect but was insufficient to guarantee the survival of all test mice, except in the test group which received 5 μg TT/immunization. (This implies that TT doses between 1 μg /immunization and 15 μg /immunization belong to the transition region.) Other doses of cholera toxin might be equally or even more beneficial, however.

Examples 83-85:

Heat labile toxin (HLT) from E.coli has as an immuno-adjuvant

Highly deformable vesicles, TransfersomesTM:

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

Heat Labile Toxin (HLT, SIGMA, Neu-Ulm), 1mg/mL plus, if required

Tetanus Toxoid (TT, pure, Accurate Antibodies) 2 mg/mL

Various relative adjuvant/antigen concentrations were used to test the effect of one of most commonly used ADP ribosylating enzymes for transcutaneous vaccination. Transfersomes were prepared as described in previous examples. Other experimental details (animal housing; antigen administration; titre determination) are described hereinabove.

Volume doses corresponding to 20 μg TT / mouse / e.c. immunization and 1 to 2 μg HLT / mouse / e.c. immunization and a positive control with 0.5 μg TT for s.c. injection were used to immunize Swiss albino mice. The results are illustrated in figure 11.

Anti-TT titres were found to increase when HLT was used as an adjuvant compared to an adjuvant free s.c. injection of antigen or compared to an adjuvant free administration of TT in transfersomes on the skin. Humoral response and the protection against a normally lethal challenge with Tetanus Toxin were found to be dose dependent, with the higher anti-TT titres and improved survival for the higher dose of HLT. The measured results (data not shown) suggest that useful HLT dosage

starts in the range 100 ng/dose while the highest practically useful dose under experimental conditions used in this test series is approx. 100-times greater. This reveals that the upper limit for adjuvant dose that should be used in conjunction with epicutaneously administered Transfersomes is of comparable order of magnitude as, and at most should be 10x higher than, that employed in conventional invasive (s.c.) immunizations; the lower practical limit, in our opinion, is 1-2 orders of magnitude smaller than the typical s.c. dose. It is proposed that similar relationships will also hold for other related immuno-adjuvants.

Examples 86-87:

Local pre-treatment with histamine

Highly deformable vesicles, Transfersomes™:

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

Tetanus Toxoid (TT, pure, Accurate Antibodies) 2 mg/mL

1 mg/ml histamine solution in phosphate buffer, 10 mM, pH 6.5

In order to test the effect of alternative means for immune-response modulation, mice were injected with 10 μ g histamine in solution at the site of e.c immunization immediately or 15 minute prior to the antigen administration on the skin in Transfersomes. The expectation was that this would induce cytokine release from the skin, and/or have some other positive effect on the outcome of non-invasive transcutaneous immunization. In order to test this hypothesis, Transfersomes were prepared as described in the previously described examples and were carried out with Swiss albino mice as described herein above (except the pre-treatment with histamine).

Figure 12 confirms the above mentioned working hypothesis. The results reveal good humoral response and the requirement for a time period between histamine injection and e.c. immunization to achieve reasonable protection against a challenge with Tetanus Toxin. Comparison with the results measured with TT in Transfersomes on

the skin without an adjuvant thus shows that histamine injection is helpful for boosting animal immune response.

Examples 88-89:Different administration routes for primary and boost immunization

Highly deformable vesicles, Transfersomes™:

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

Cholera Toxin (CT, SIGMA, Neu-Ulm), 0-1 µg/immunisation

Tetanus Toxoid (TT, pure, Accurate Antibodies) 2 mg/mL

A detailed description of vesicle preparation and animal experimentation can be found in previous examples hereinabove.

Volume doses corresponding to 20 µg TT alone or to 0.5 µg TT plus 1 µg CT were injected s.c. for primary immunization but were applied epicutaneously for booster immunization. This combination increased the efficacy of vaccination substantially, as implied earlier.

Anti-TT titres were high enough to yield 100% protection against a challenge with Tetanus Toxin (Figure 13). Comparison with the data from previous examples carried out with adjuvant free TT-Tfs on skin clearly demonstrate the usefulness of combined s.c./e.c. vaccination protocol.

The data therefore suggest that antigen-loaded Transfersome™ applied on the skin provide an attractive complement to invasive antigen administration, which should be particularly important for the purpose of booster immunization.

Example 90:Bi-valent vaccination with Tetanus Toxoid and Cholera Toxin as antigens

Highly deformable vesicles, Transfersomes™:

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

Cholera Toxin (CT, SIGMA, Neu-Ulm), 10 μ g/immunization

Tetanus Toxoid (TT, pure, Accurate Antibodies) 2 mg/mL

Cholera Toxin not only works as an adjuvant to improve anti-TT response (see previous examples), but is also an antigen by itself. The adjuvant, consequently, can play the role of a secondary antigen, when used at certain concentration. The adjuvancy and immunogenicity are not linearly correlated, however, which offers interesting possibilities for optimizing the outcome of vaccination outcome (also with regard to side effects and allergy induction.) Working with CT in Transfersomes tested in mice, we found out that CT doses between less than 50 ng and at least 10 μ g per application are useful for the purposes described in this application.

Anti-cholera toxin antibodies in the mice that were treated with different volumes of test formulation on the skin (corresponding to 10 μ g TT and 10 μ g CT) are indicative of CT antigenicity. This corroborates the potential of ultradeformable vesicles for making at least bi-valent vaccines based on Transfersomes™ containing more than one antigen.

Figure 14 shows TT and CT titres measured with the mice that were previously immunized with TT and CT in the same carrier.

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CLAIMS

1. A transdermal vaccine comprising
 - (a) a transdermal carrier which is a penetrant, suspended or dispersed in an aqueous solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance, and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains;
 - (b) a compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity itself; and
 - (c) an antigen, an allergen, a mixture of antigens and/or a mixture of allergens.
2. The vaccine according to claim 1, wherein the compound displaying or inducing cytokine or anti-cytokine activity and the antigen are associated with the penetrant.
3. The vaccine according to any one of claims 1 or 2, wherein the less soluble self-aggregating molecule is a polar lipid and the more soluble component is a

surfactant or a surfactant-like molecule or else such form of polar lipid which is sufficiently soluble for the purpose of this invention.

4. The vaccine according to any one of claims 1 to 3, wherein the average diameter of the penetrant is between 30 nm and 500 nm, preferably between 40 nm and 250 nm, even more preferably between 50 nm and 200 nm and particularly preferably between 60 nm and 150 nm.
5. The vaccine according to any one of claims 1 to 4, wherein the total weight of droplets in the formulation for the use on human or animal skin is 0.01 weight-% (w-%) to 40 weight-% of total mass, in particular between 0.1 w-% and 30 w-%, and most preferably between 5 w-% and 20 w-%.
6. The vaccine according to any one of claims 1 to 5, wherein total antigen concentration is between 0.001 and 40 w-% of the total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 20 w-% and most preferably between 0.5 w-% and 10 w-%.
7. The vaccine according to any one of claims 1 to 6 further comprising
 - (da) a low molecular weight chemical irritant; and/or
 - (db) an extract or a compound from a pathogen or a fragment or a derivative thereof.
8. The vaccine according to any one of claims 1 to 7 wherein the compound exerting cytokine activity is IL-4, IL-3, IL-2, TGF, IL-6, IL-7, TNF, IL-1 α and/or IL-1 β , IL-12, IFN- γ , TNF- β , IL-5 or IL-10 a type I interferon, preferably IFN-alpha or IFN- β .
9. The vaccine according to any one of claims 1 to 8 wherein the compound displaying anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative or an analogue thereof.
10. The vaccine according to any one of claims 1 to 9 wherein the antigen is derived from a pathogen.

11. The vaccine according to claim 10 wherein said pathogen is selected from extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus*, gram-negative bacteria, such as *Meningococcus* and *Gonococcus* species, species of *Neisseria*, gram negative bacteria, including enteric organisms such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Diphtheria*, *Bordetella Pertussis*, and gram-positive bacteria (e.g. *Bacillus pestis*, *BCG*), particularly anaerobes, such as the *Clostridium* species (e.g. *Clostridium tetani*, *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*), bacteria and viruses, which survive and replicate within host cells, comprising mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes*, retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species (e.g. *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae*, *B. ovis*), the causative agent for cholera (e.g. *Vibrio cholerae*), *Haemophilus* species like *H. actinomycetemcomitans*, *H. pleuropneumoniae*, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases and pathogens that cause various neoplasiae, auto-immune diseases or are related to other pathological states of the animal or human body which do not necessarily result from pathogen infections.
12. The vaccine according to any one of claims 1 to 11, wherein the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.
13. The vaccine according to any of claims 1 to 12, wherein the concentration of each compound displaying cytokine activity used is selected to be up to 1000 times higher than the concentration optimum established in the corresponding tests with the antigen dose and immunoadjuvant chosen, performed by injecting

the formulation or performing the tests *in vitro*, and preferably is up to 100x, more often up to 50x and even better up to 20x higher.

14. The vaccine according to any one of claims 7 to 13, wherein the pathogen extract or compound is a lipopolysaccharide, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a pathogen; an extract of a pathogen, including bacterial exo- and endotoxins, preferably cholera toxin and the heat labile toxin of *E. coli*, an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, or a purified protein derivative of *M. tuberculosis*, LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes*, or outer membrane protein of group B *Neisseria meningitidis* (GBOMP); or bacterial or viral nucleic acids such as oligonucleotides containing unmethylated CpG dinucleotides.
15. The vaccine according to claim 14 wherein said lipopolysaccharide is lipid A or a derivative and modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose.
16. The vaccine according to any one of claims 7 to 13, wherein the concentration of the pathogen compound derived from a pathogen is between 10x lower and up to 1000x higher than that otherwise used with the corresponding injected formulations employing similar antigen, the epicutaneously administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.
17. The vaccine according to any one of claims 7 to 16 wherein said low molecular weight irritant is selected from the classes of allergenic metal ions, acids, bases, irritating fluids, (fatty-) alcohols, (fatty-) amines, (fatty-) ethers, (fatty-) sulphonates, -phosphates, etc., or other suitable solvents or amphiphiles, or from the group of surfactant-like molecules, often with the skin permeation enhancing capability, as well as derivatives or combinations thereof.

18. The vaccine according to any one of claims 7 to 17, wherein the concentration of a low molecular weight irritant is chosen to be by at least the factor of 2, more often by the factor of 5, and even better by the factor of 10 or more, below the concentration which in independent tests on the same or a comparable subject is deemed to be unacceptable owing to the local irritation, as assessed by the methods and standards commonly used to test such an irritant.
19. The vaccine according to any one of claims 7 to 16 wherein the allergen belongs to the class of the inhalation allergens, including but not limited to various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; furthermore, food and drug allergens; contact allergens; injection, invasion or depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., a part of implantation material.
20. The vaccine according to any one of claims 1 to 19, wherein the applied dose of an antigen differs by the factor of 0.1 to 100 from the dose which otherwise would have to be injected in the process of immunisation, but more often is in the range between 0.5 to 50, even better between 1 and 20 and ideally is less than 10x higher than that used with an injection.
21. The vaccine according to any one of claims 1 to 20, wherein the applied penetrant dose is between 0.1 mg cm^{-2} and 15 mg cm^{-2} , even more often is in the range 0.5 mg cm^{-2} and 10 mg cm^{-2} , and preferably is between 1 mg cm^{-2} and 5 mg cm^{-2} .
22. The vaccine according to any one of claims 1 to 21 wherein said antigen is a pure or purified antigen.
23. A kit comprising, in a bottled or otherwise packaged form, at least one dose of the vaccine according to any one of claims 1 to 22.
24. The kit according to claim 23 further comprising at least one injectable dose of the antigen specified in claim 11 or of the allergen specified in claim 12.

25. A method for generating a protective immuno response on a mammal by vaccinating said mammal with a vaccine according to any one of claims 1 to 22.
26. The method according to claim 25 wherein different treatment areas are selected to control the applied immunogen dose and the outcome of therapeutic vaccination.
27. The method according to claim 25 or 26, wherein a suspension of antigen-free penetrants is loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the administration of resulting formulation on the skin.
28. The method according to any one of claims 25 to 27, wherein the vaccine of any one claims 1 to 22 is applied on the skin after pre-treating the organ by an immunoadjuvant manipulation, said manipulation comprising, for example, skin rubbing, pressing, heating, exposing to an electrical or mechanical, e.g. ultrasound field, etc., or injecting a non-immunogenic formulation (like histamine) in the skin, provided that any such treatment releases immunoadjuvant compounds from the skin or other peripheral immuno-active tissues or else reduces the concentration/duration of action of antagonists to the desired vaccination.
29. The method according to any one of claims 25 to 28 wherein immunogen is applied in a non-occlusive patch.
30. The method of any one of claims 25 to 29 characterized in that at least one dose of vaccine is administered.
31. The method according to claim 30 wherein said vaccine is administered as a booster vaccination.
32. The method according to claim 31, wherein the primary immunisation is done

invasively, typically using a subcutaneous injection or some other suitable skin barrier perforating/destroying method, and wherein the at least one subsequent, booster immunisation is done non-invasively.

33. The method according to any one of claims 25 to 32, wherein the vaccine is applied between 2 and 10, preferably between 2 and 7, even more preferably up to 5 and most preferably up to 3 times, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined according to a suitable assessment method, or else to deem the effort as having failed.
34. The method according to claim 33, wherein the time interval between the subsequent vaccinations is chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years.
35. The method according to any one of claims 25 to 34, wherein the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is determined as a function of a suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimize the formulation or application further.
36. Use of the transdermal carrier, the compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity, the antigen or allergen, and optionally an extract or a compound from a microorganism or a fragment or a derivative thereof, and/or a low molecular weight chemical irritant as defined in any one of the preceding claims for the preparation of a vaccine for inducing a protective or tolerogenic immune response.

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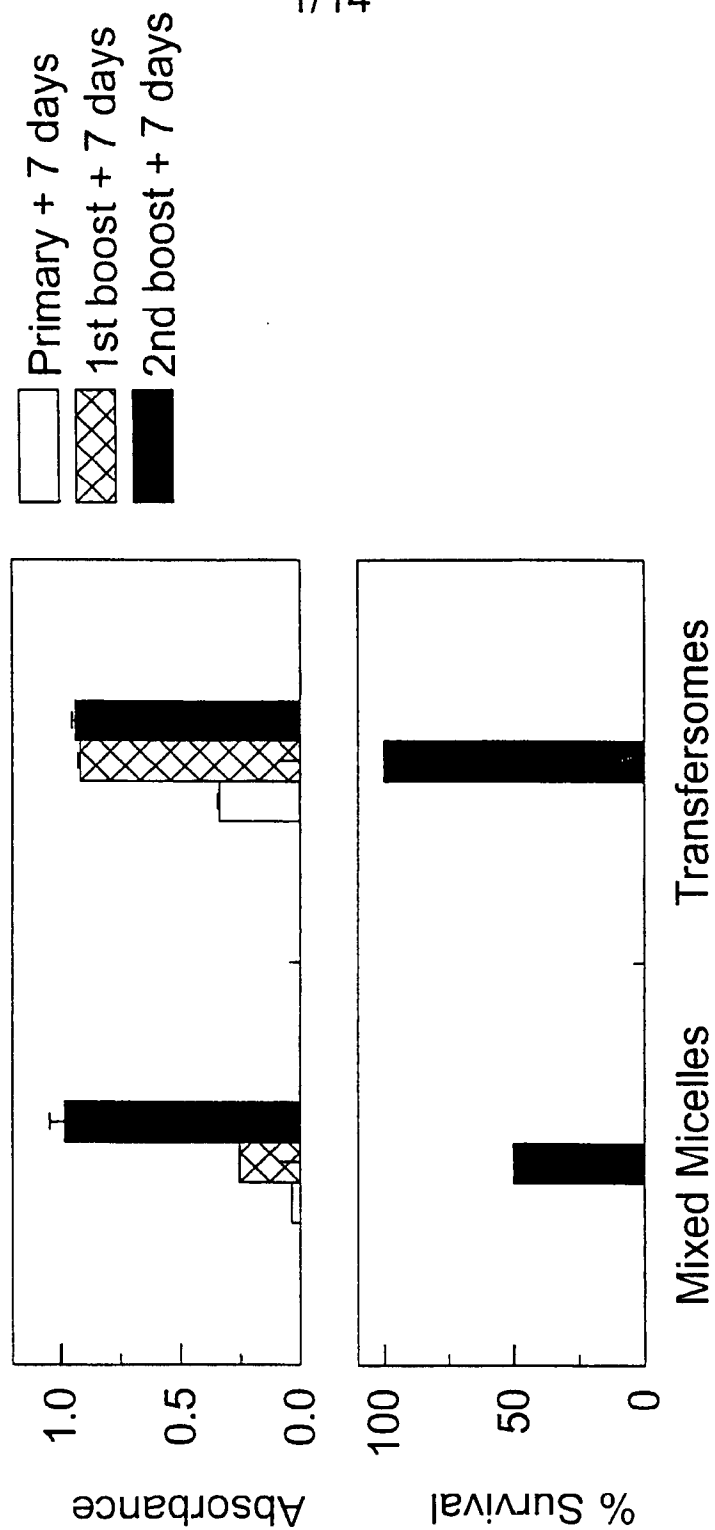


Fig. 1

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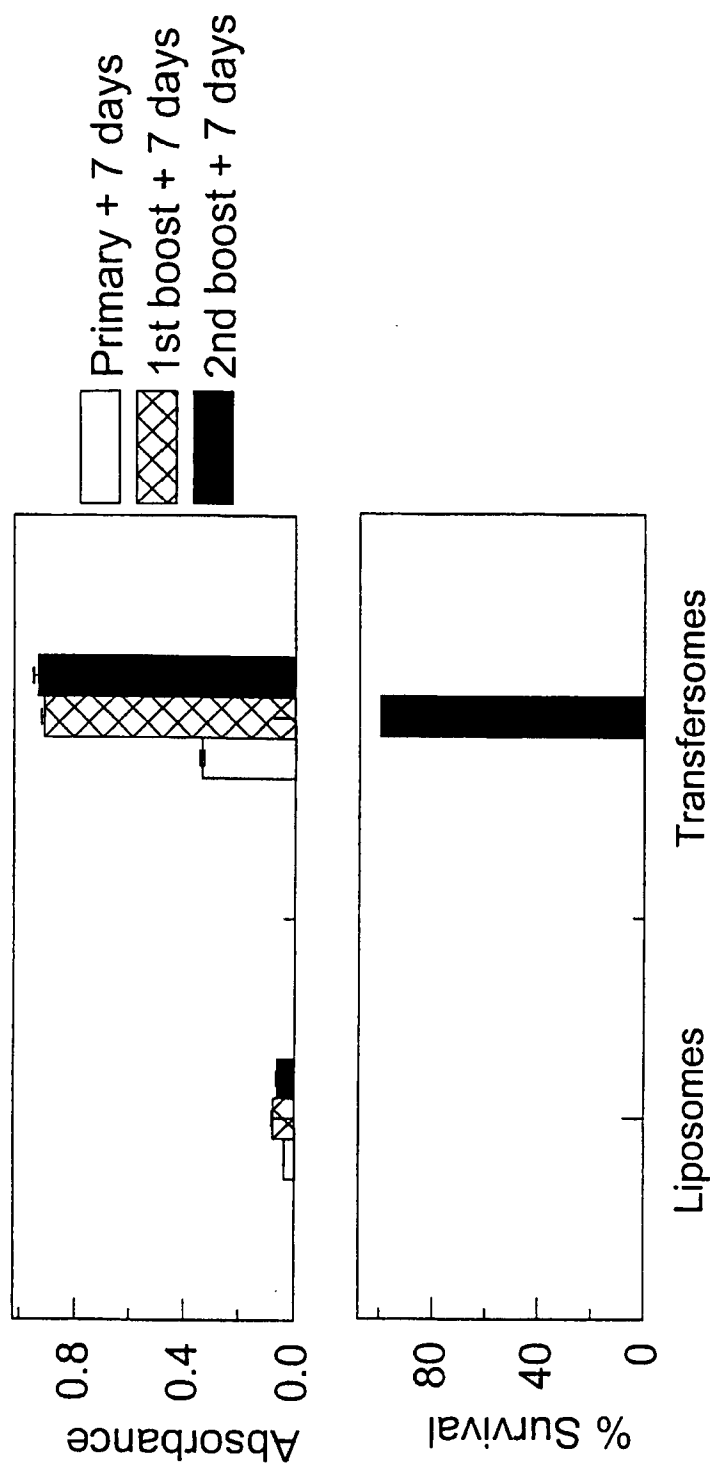


Fig. 2

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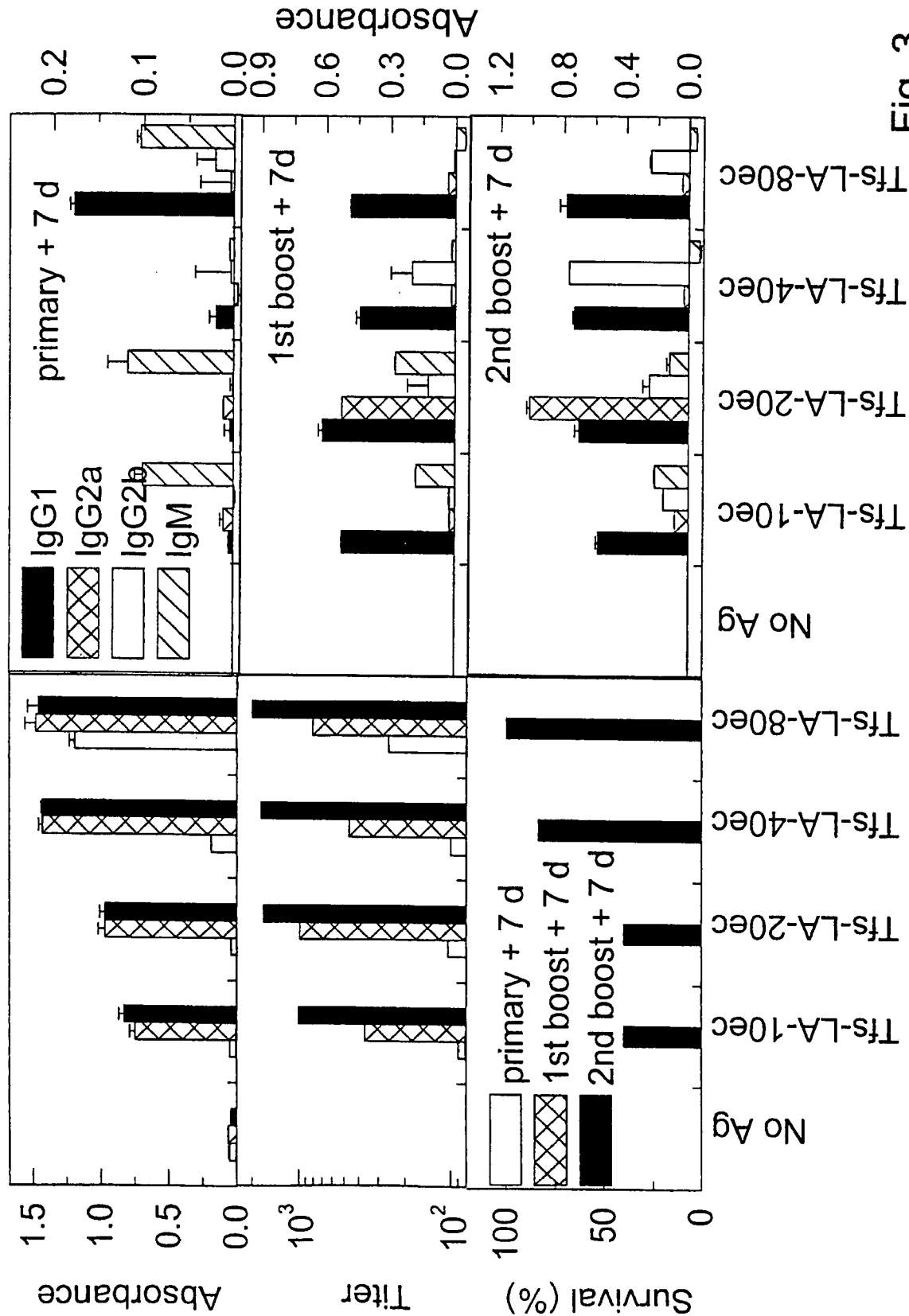


Fig. 3

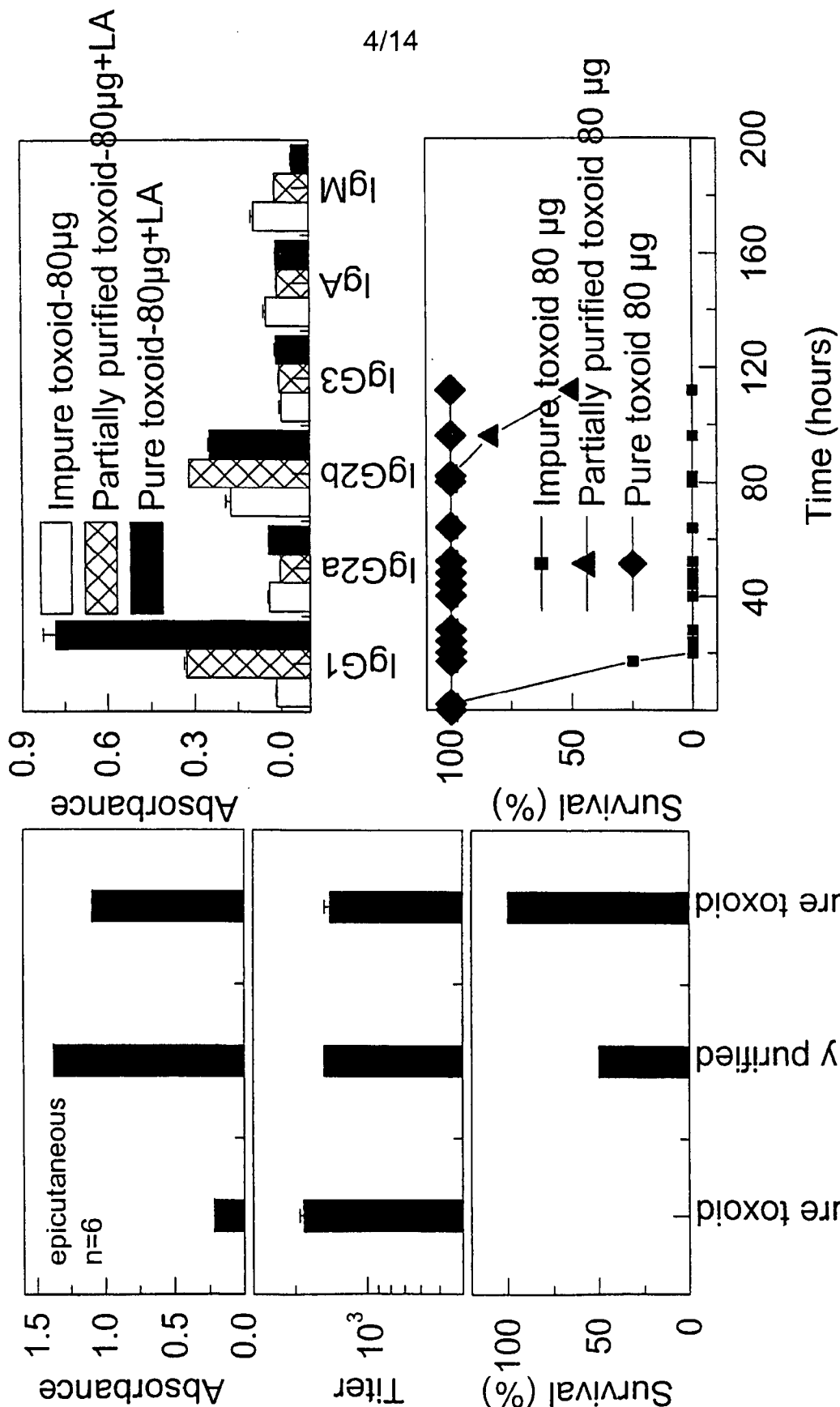


Fig. 4

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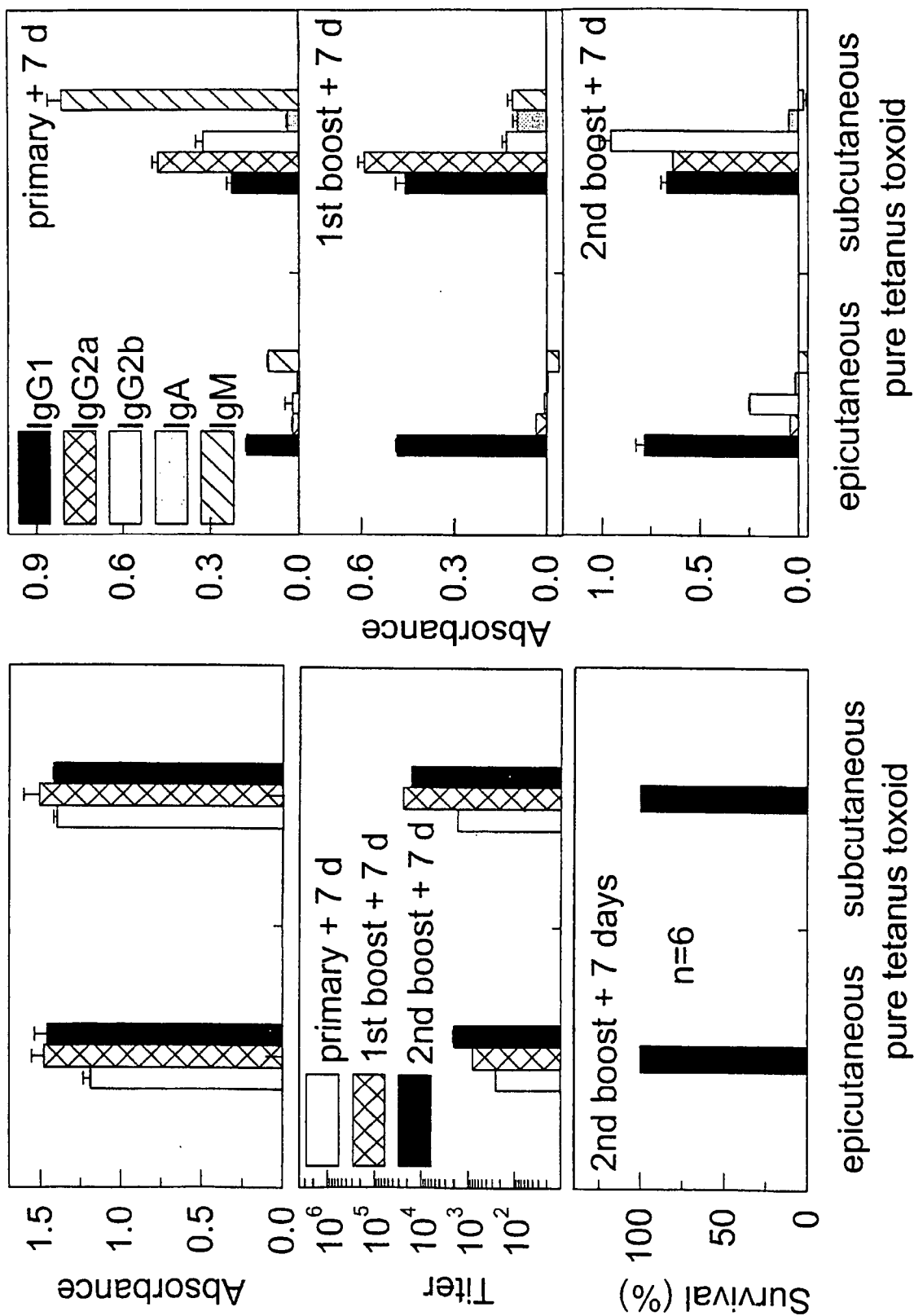


Fig. 5

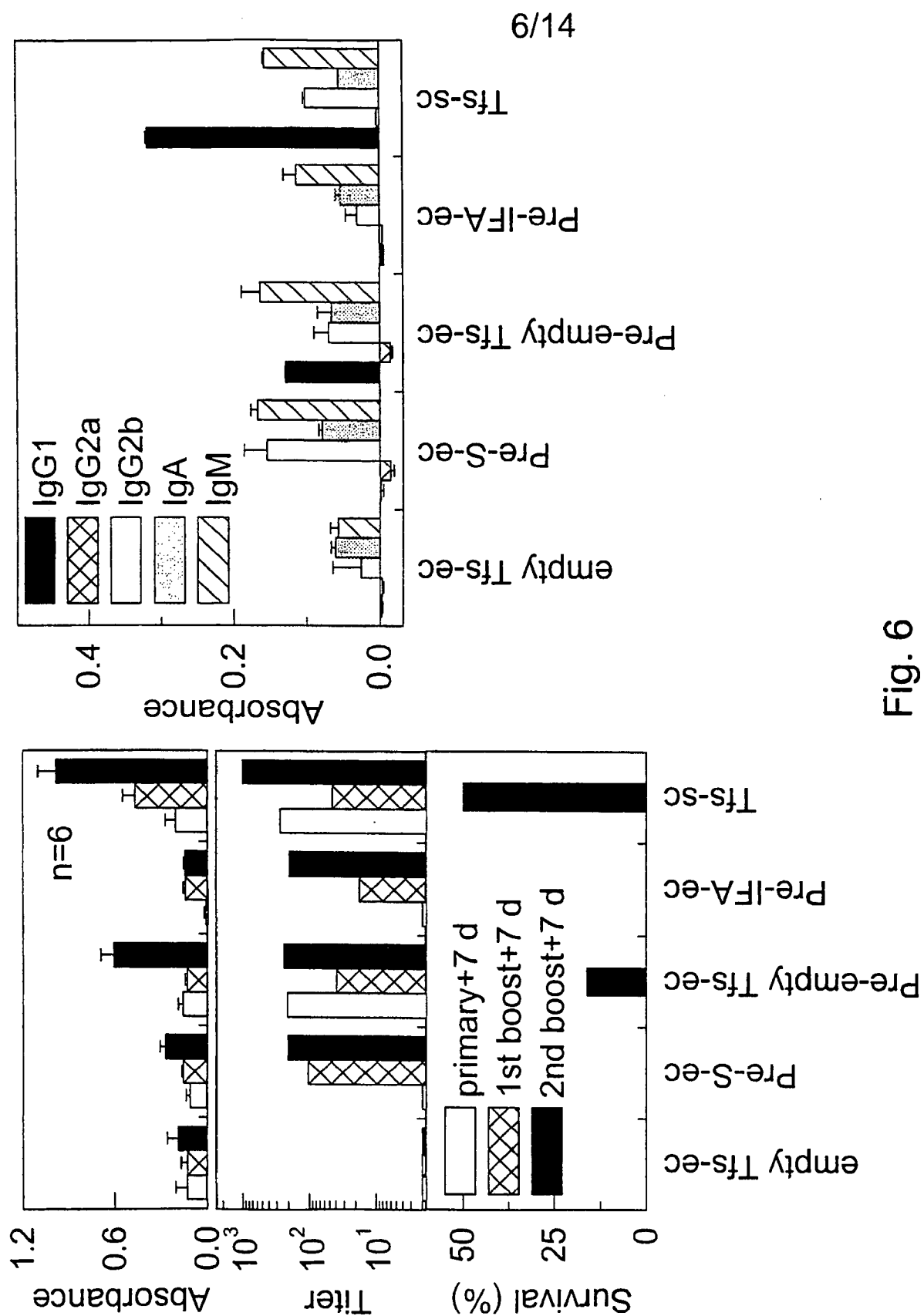


Fig. 6

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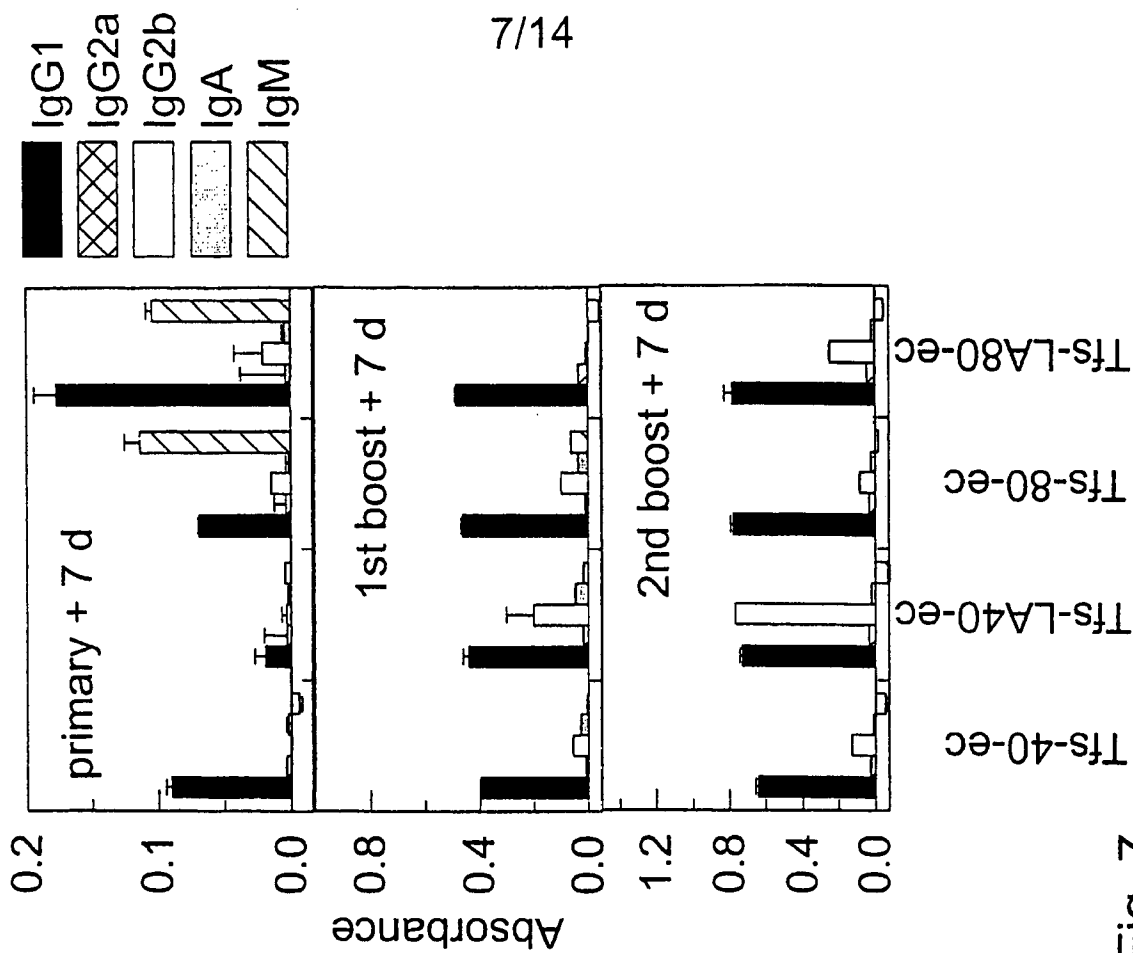
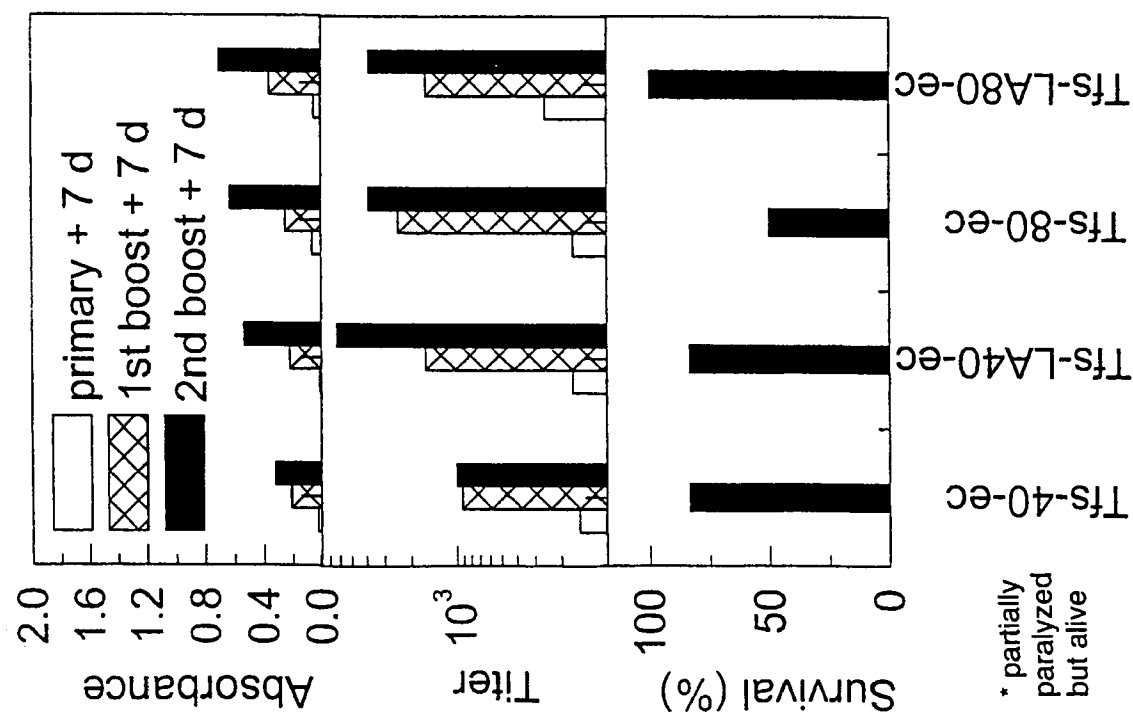


Fig. 7



* partially paralyzed but alive

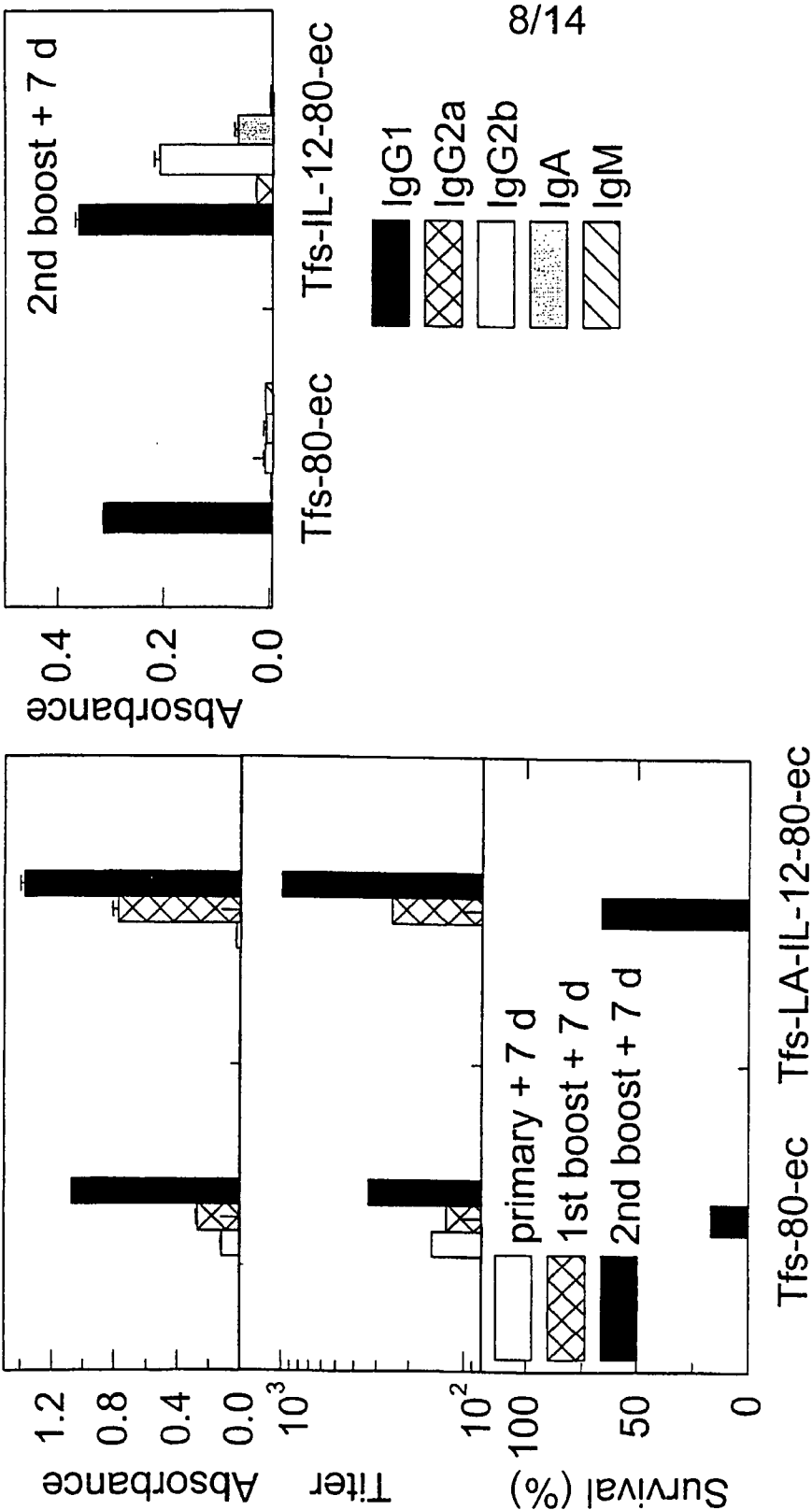


Fig. 8

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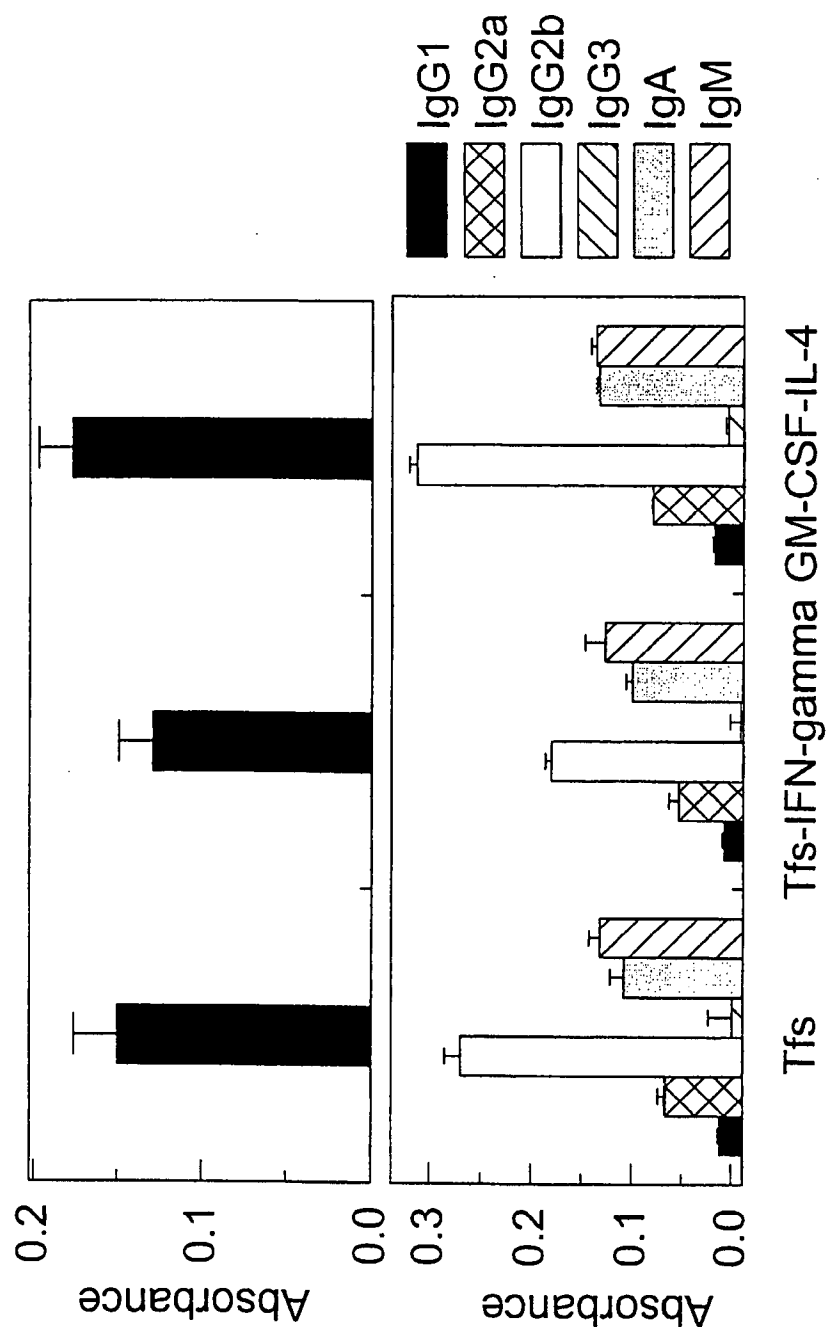
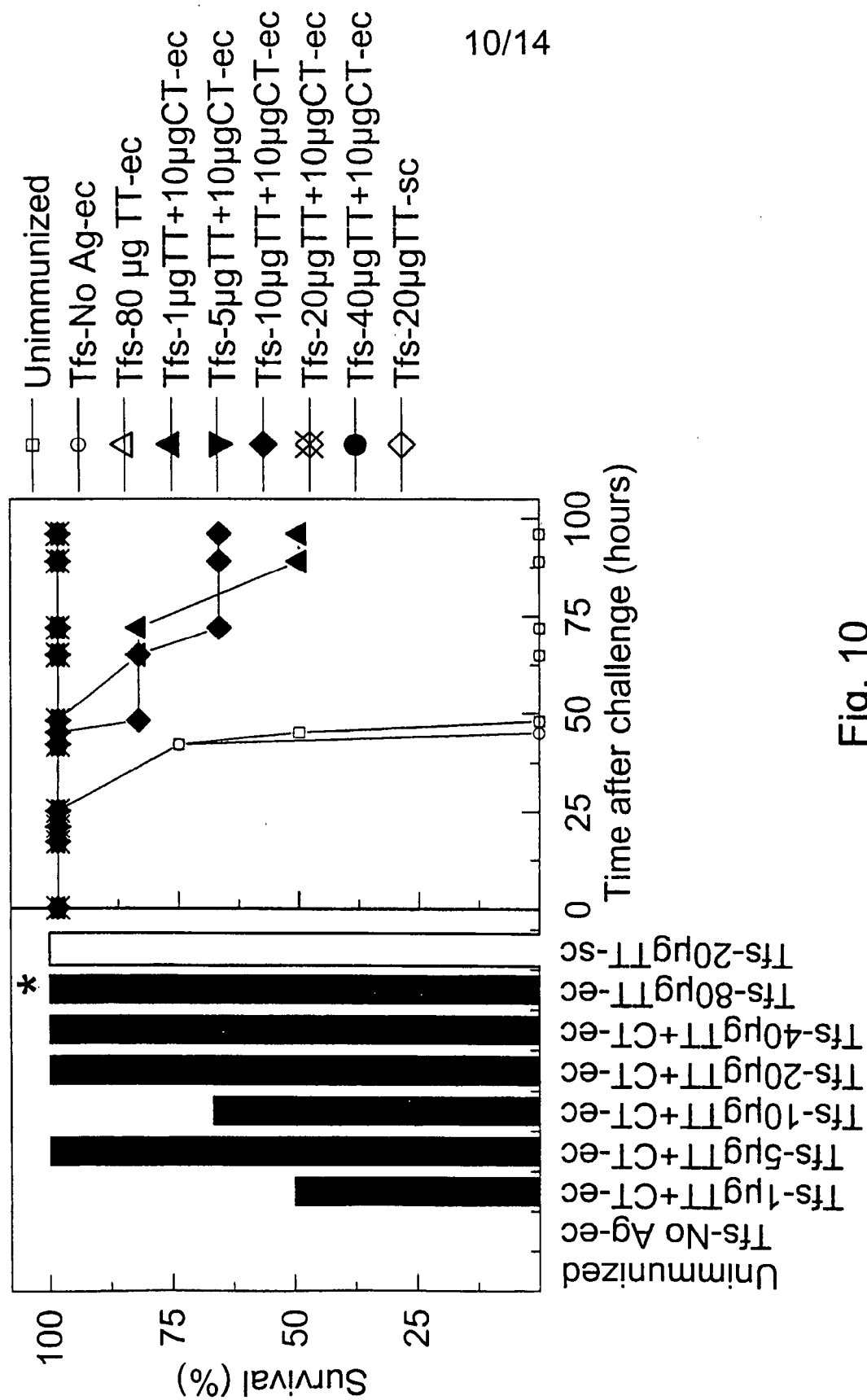


Fig. 9



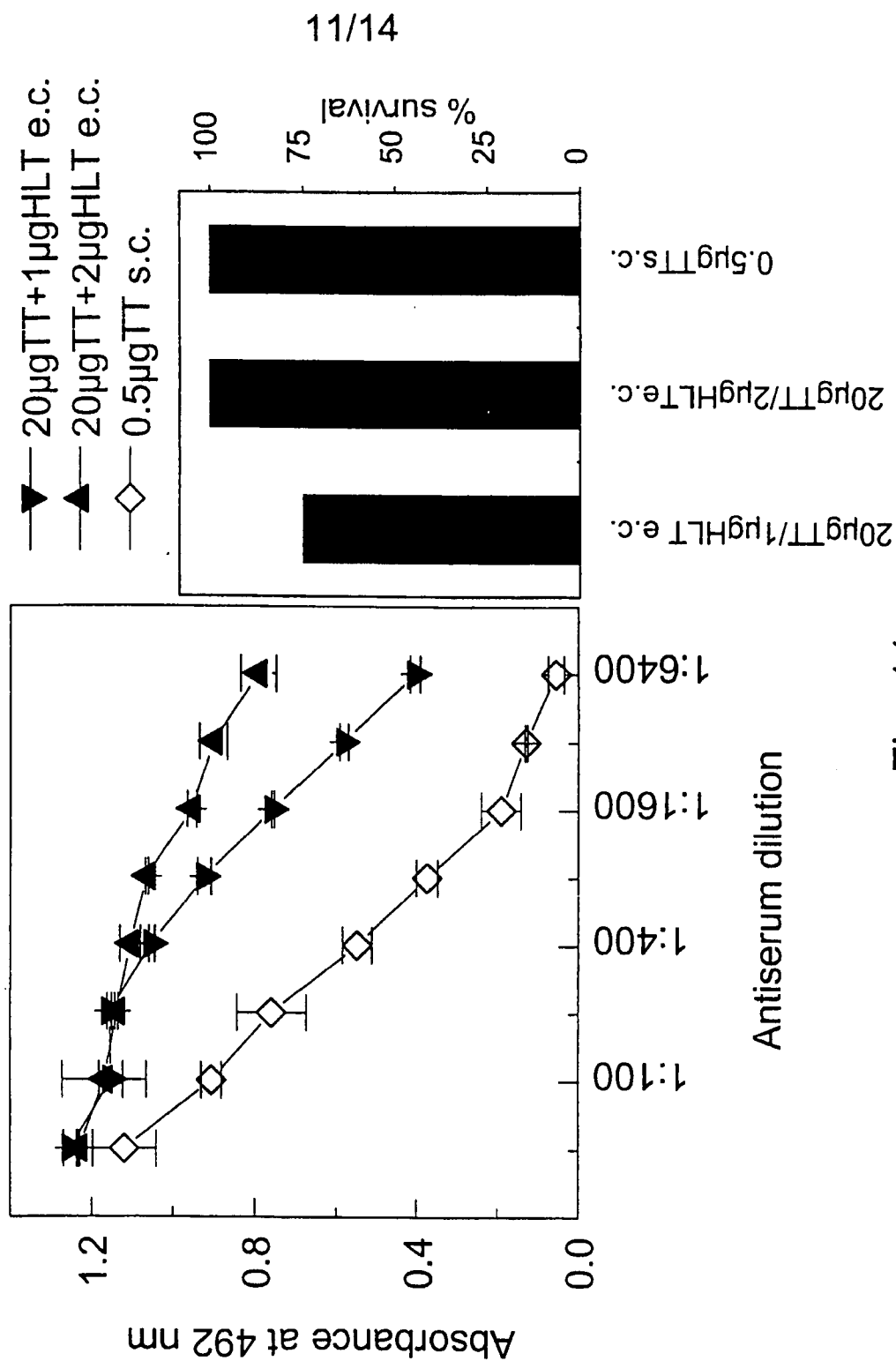


Fig. 11

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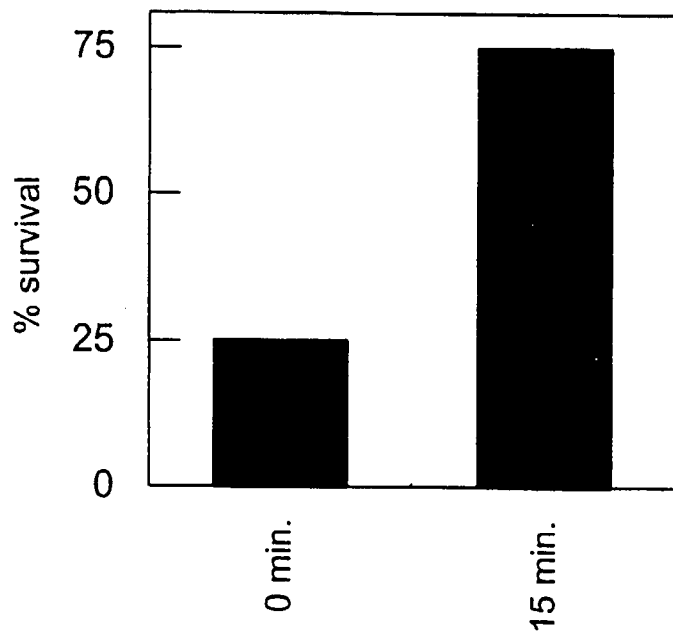
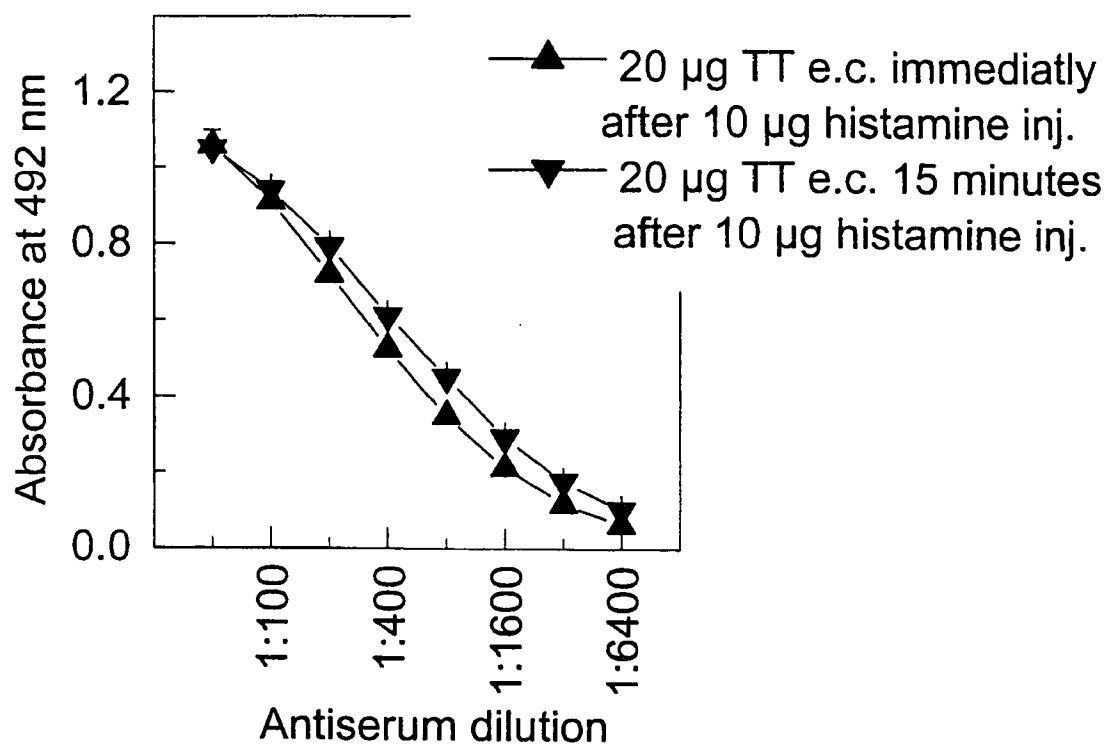


Fig. 12

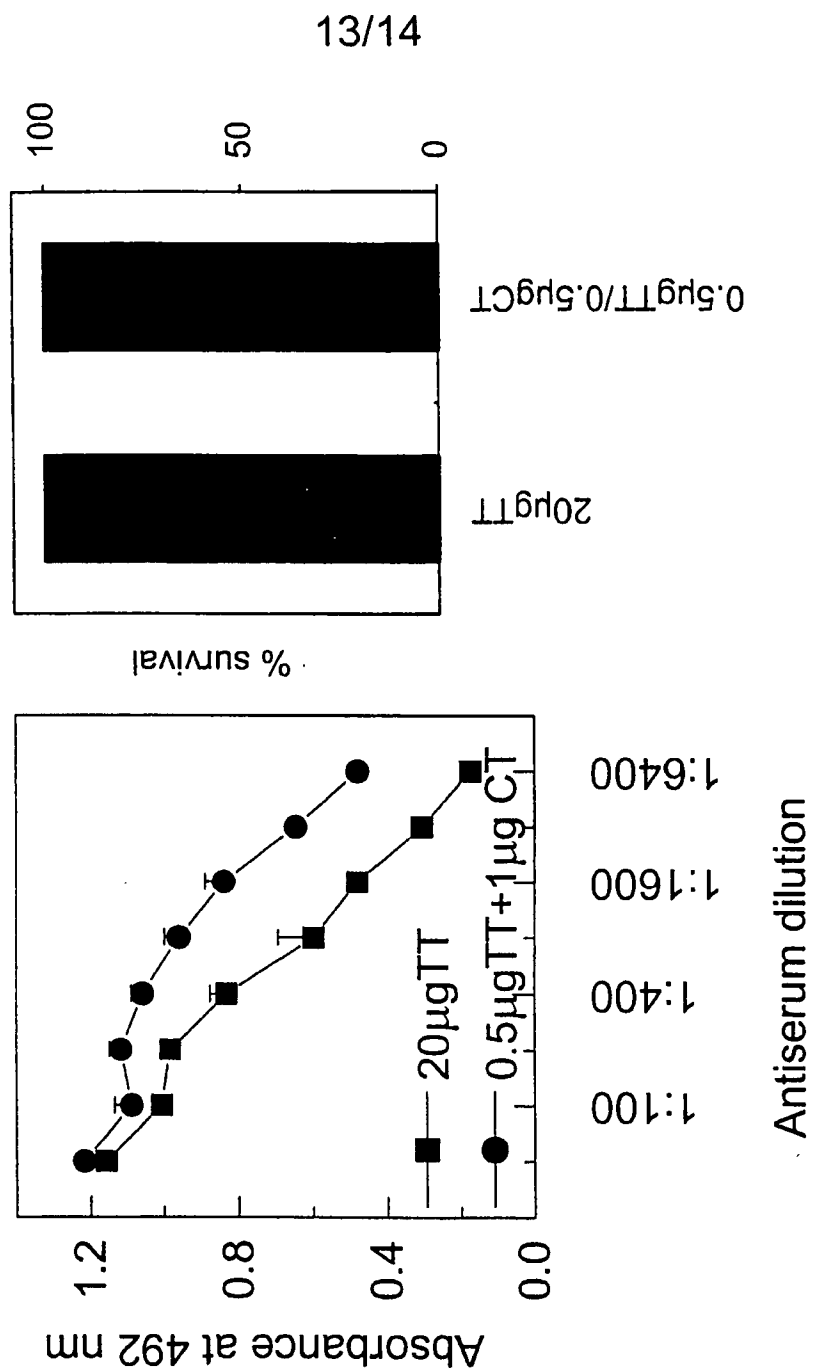


Fig. 13

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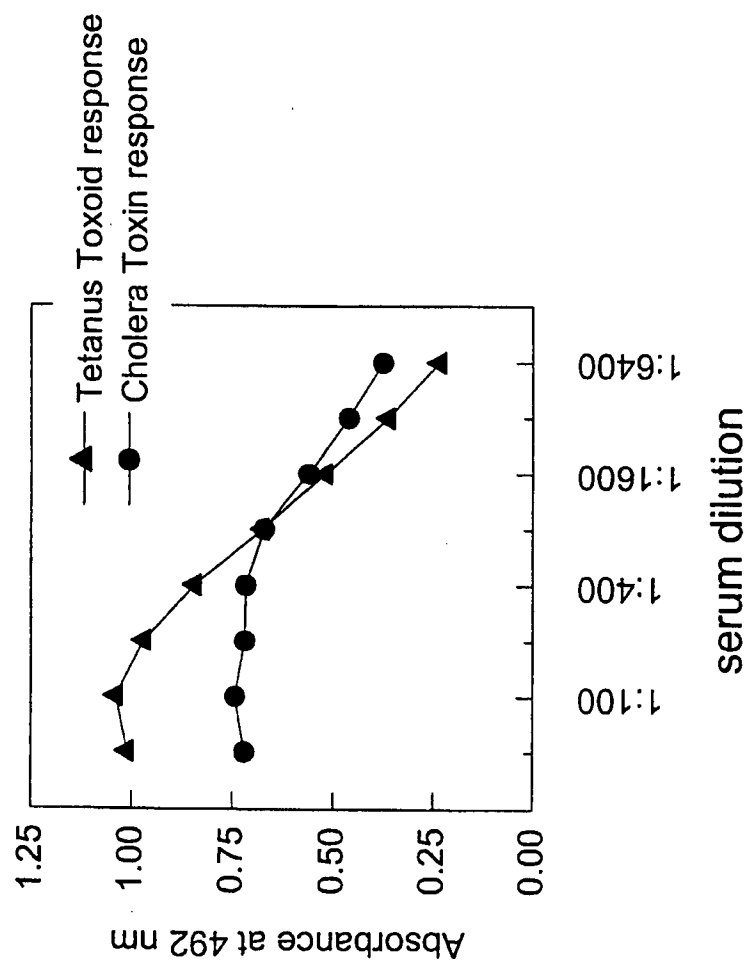


Fig. 14

INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/EP 00/00597

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K9/127 A61K38/19 A61K39/39 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PAUL A, CEVC G: "Non-invasive administration of protein antigens: transdermal immunization with bovine serum albumin in transfersomes" VACCINE RESEARCH, vol. 4, no. 3, 1995, pages 145-164, XP002107365 cited in the application abstract page 147, last paragraph -page 149, paragraph 1 page 153, paragraph 2; figure 5 page 159, paragraph 1 page 162, last paragraph -page 163, paragraph 1</p> <p style="text-align: center;">--- -/-</p>	<p>1-7, 10-16, 19-23, 25,26, 28,30, 31,33, 35,36</p>

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.*** Special categories of cited documents :**

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Date of the actual completion of the international search

19 May 2000

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Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/EP 00/00597

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PAUL A ET AL: "Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes"</p> <p>VACCINE, vol. 16, no. 2-3, 2 January 1998 (1998-01-02), page 188-195 XP004098622 cited in the application abstract * page 189, paragraph "Immunogen preparation" * page 194, column 1, line 33 -column 2, line 15</p>	1
A	<p>CEVC G: "Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery"</p> <p>CRITICAL REVIEWS IN THERAPEUTIC DRUG CARRIER SYSTEMS, vol. 13, no. 3-4, 1996, pages 257-388, XP002107366 page 316 -page 321</p>	1
A	<p>WO 91 01146 A (PRAXIS BIOLOG INC) 7 February 1991 (1991-02-07) page 3, line 10 -page 4, line 13 page 9, line 17-21 page 10, line 5-11 claims</p>	1-36
A	<p>WO 92 04009 A (UNIV LONDON PHARMACY) 19 March 1992 (1992-03-19) page 1, line 3-7 page 3, line 21-32 page 6-7; example 1 page 14; example 2 claims</p>	1-36
A	<p>GLENN G M ET AL: "Skin immunization made possible by cholera toxin 'letter!'"</p> <p>NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 391, no. 6670, 26 February 1998 (1998-02-26), page 851 XP002110053 ISSN: 0028-0836 cited in the application</p>	1-36

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 00/00597

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 25-35 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/EP 00/00597

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9101146 A	07-02-1991	AU 651949 B	11-08-1994
		AU 6055090 A	22-02-1991
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(57) 要約

緊急ブレーキ操作が行われた際に大きな制動力を発生させるBA制御と、車輪のスリップ率を抑制するABS制御の双方を実現する制動力制御装置において、BA制御とABS制御の干渉が防止される。BA制御の開始と判定された際に（ステップ100, 102）フロント1輪以上についてABS制御が実行されている否かを判別する（ステップ104）。フロント1輪以上についてABS制御が実行されている場合は、BA制御が開始されると同時に多量の液圧が後輪のホイールシリンダに供給されると判断し、その流入を抑制するBA勾配抑制制御を開始する（ステップ108）。フロント輪でABS制御が実行されていない場合は通常のBA制御を開始する（ステップ106）。

参考情報

PCTに基づいて公開される国際出願のパンフレット第一頁に記載されたPCT加盟国を特定するために使用されるコード

AL	アルバニア	ES	スペイン	LR	リベリア	SG	シンガポール
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明細書

制動力制御装置

5

技術分野

本発明は、制動力制御装置に係り、特に、自動車用制動装置によって発生される制動力を制御する装置として好適な制動力制御装置に関する。

10

背景技術

特開平 4 - 1 2 1 2 6 0 号は、ブレーキアシスト機能及びアンチロックブレーキ機能を有する制動力制御装置を開示している。ブレーキアシスト機能（以下、ABS機能と称す）は、運転者によって緊急ブレーキ操作が行われた際に通常時に比して大きな制動油圧を発生させるために設けられる。アンチロックブレーキ機能（以下、ABS機能と称す）は、ブレーキ操作の実行中に各車輪に過剰なスリップ率が生じないように各車輪のホイールシリンダ圧 $P_{w/c}$ を制御するために設けられる。

20

ABS機能を実現する従来の制動力制御装置は、ブレーキ踏力に応じた制動液圧を発生するマスタシリンダと、マスタシリンダと各車輪との導通状態およびリザーバタンクと各車輪の導通状態を制御する液圧回路とを備えている。液圧回路は、ホイールシリンダ圧 $P_{w/c}$ を増圧する必要があるホイールシリンダをマスタシリンダに導通させ、ホイールシリンダ圧 $P_{w/c}$ を減圧する必要があるホイールシリンダをリザーバタンクに導通させるように制御される。上記の制御によれば、各車輪のホイールシリンダ圧 $P_{w/c}$ をマスタシリンダの発する制動液圧に比して低い領域で適当に制御することができる。

25

BA機能を実現する従来の制動力制御装置は、ブレーキ踏力と無関係に所定の液圧を発生する高圧源と、高圧源の発する液圧を適当

- に減圧制御して各車輪のホイールシリンダに供給する液圧制御弁とを備えている。液圧制御弁は、運転者によって緊急ブレーキ操作が実行されていない場合は、ブレーキ踏力に対して所定の倍力比で昇圧された制動液圧を各車輪に対して供給する。また、液圧制御弁は、
- 5 運転者によって緊急ブレーキ操作が実行された場合は、高圧源の発する最大の液圧のブレーキ液を各車輪のホイールシリンダに供給する。

上記の処理によれば、運転者によって通常のブレーキ操作が実行されている場合は、各車輪のホイールシリンダに、ブレーキ踏力に応じたホイールシリンダ圧 $P_{w/c}$ を供給することができる。また、運転

10 者によって緊急ブレーキ操作が実行された場合は、各車輪のホイールシリンダに、通常時に比して高圧のホイールシリンダ圧 $P_{w/c}$ を供給することができる。このように、上記従来の制動力制御装置によれば、通常ブレーキとしての機能と B A 機能とを適切に実現することができる。

- 15 B A 機能と A B S 機能とを共に実現する制動力制御装置としては、例えば、液圧回路の上流側に、マスタシリンダの発する制動液圧と、高圧源の発する制動液圧とを選択的に供給し得る装置が考えられる。かかる制動力制御装置において、A B S 機能は、マスタシリンダにより発生する液圧を液圧回路に供給しつつ、上述した手法で液圧回路
- 20 路を制御することにより実現される。また、B A 機能は、マスタシリンダと液圧回路とを遮断した状態で、液圧回路を介して、高圧源により発生する制動液圧を各車輪のホイールシリンダに供給することにより実現される。以下、上記の制動力制御装置において B A 機能を実現するための制御を B A 制御と、また、A B S 機能を実現する
- 25 ための制御を A B S 制御と称す。

B A 機能と A B S 機能とを共に実現する上記の制動力制御装置において、B A 制御が開始されると、何れかの車輪に過剰なスリップ率が生ずることがある。この場合、その車輪について A B S 制御を実行すれば、B A 機能と A B S 機能とを同時に実現することができ

る。かかる機能は、液圧回路に対して高圧源により発生する制動液圧を供給しつつ、過剰なスリップ率の発生した車輪のホイルシリンダが適宜リザーバタンク側へ接続されるように液圧回路を制御することで実現できる。以下、上記の機能をBA+ABS機能と、また、

5 BA+ABS機能を実現するための制御をBA+ABS制御と称す。

上述したBA+ABS制御によれば、運転者によって緊急ブレーキ操作が実行された後に、過剰なスリップ率の発生した車輪のホイルシリンダ圧 $P_{w/c}$ をABS制御の要求に応じて増減しつつ、他の車輪のホイルシリンダ圧 $P_{w/c}$ をBA制御の要求に応じて増圧する

10 ことができる。

しかし、BA+ABS制御の実行中は、BA制御とABS制御とに干渉が生ずる。すなわち、BA+ABS制御の実行中は、BA制御が単独で実行される場合、および、ABS制御が単独で実行される場合と異なる環境が形成される。このため、BA+ABS制御を

15 実行するにあたり、BA制御およびABS制御が、それらが単独で実行される場合と同様に実行されると、ホイルシリンダ圧 $P_{w/c}$ を適正に制御できない事態が生じ得る。

つまり、BA+ABS制御の実行中は、ABS制御の対象車輪（以下、ABS対象車輪と称す）のホイルシリンダを、その車輪に

20 ついてホイルシリンダ圧 $P_{w/c}$ の増圧が要求される僅かな時間を除き、高圧源から切り離す必要がある。一方、高圧源には、BA制御が開始された後、4つの車輪全てのホイルシリンダ圧 $P_{w/c}$ を適切な増圧勾配で増圧させるに足る能力が与えられている。このため、BA+ABS制御の実行中は、ABS制御の非対象車輪（以下、ABS非対象車輪と称す）に対するホイルシリンダ圧 $P_{w/c}$ の変化率は、BA制御が単独で実行される場合に比して急激な増圧勾配となる。

25

また、ABS制御が単独で実行されている場合は、ABS対象車輪のホイルシリンダに、マスタシリンダ圧 $P_{m/c}$ が供給される。こ

れに対して、BA+ABS制御の実行中は、ABS対象車輪のホイールシリンダに、高圧源の発する液圧が供給される。高圧源は、マスタシリンダ圧 $P_{m/c}$ として通常生ずる液圧に比して高い液圧を発生する。このため、BA+ABS制御の実行中は、ABS制御が単独
5 で実行される場合に比して、ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ の変化率が急激な増圧勾配になり易い。

ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ は、その車輪に過剰なスリップ率が発生した時点で減圧され、その後、比較的緩やかに増圧される。この増圧の際に、ホイールシリンダ圧 $P_{w/c}$ が急激な増圧
10 勾配で増加されるとすれば、ホイールシリンダ圧 $P_{w/c}$ の増圧が開始された後、即座にそのホイールシリンダ圧 $P_{w/c}$ を減圧する必要がある。このため、BA+ABS制御の実行中に、上記の如くホイールシリンダ圧 $P_{w/c}$ が急激な増圧勾配で増圧されるとすれば、ABS対象車輪について制御上のハンチングが生じ易くなる。

15 このように、上述した手法によってBA+ABS機能を実現しようとした場合、BA制御とABS制御とが互いに干渉し合い、ABS対象車輪に制御上のハンチングが生じ易く、また、ABS非対象車輪に（BA制御の対象車輪に）過剰な増圧勾配が発生するという問題がある。この点、上述した手法は、BA+ABS機能を実現す
20 るうえで、必ずしも最適な手法ではない。

発明の開示

本発明の総括的な目的は、上述の問題を解決した改良された有用な制動力制御装置を提供することである。

25 本発明のより具体的な目的は、BA制御とABS制御との干渉を防止して、適切にBA+ABS機能の両方を実現することのできる制動力制御装置を提供することである。

上記の目的を達成するために、本発明の一つの面によれば、ホイールシリンダへの液圧流入経路を遮断した状態でホイールシリンダ圧を

制御する制動液圧減圧制御と、運転者によって緊急ブレーキ操作が実行された際に通常時に比して大きな制動液圧を発生させるブレーキアシスト制御とを実行する制動力制御装置において、

5 ホイルシリンダの液圧流入経路の導通状態を検出する導通検出手段と、

前記ブレーキアシスト制御の開始時に、何れかのホイルシリンダの液圧流入経路が実質的に遮断されている場合には、他のホイルシリンダへの制動液圧の流入を抑制する液圧流入抑制手段と、

を備える制動力制御装置が提供される。

10 上述の発明において、何れかのホイルシリンダについて制動液圧減圧制御が実行されている場合は、そのホイルシリンダへの制動液圧の流入が阻止される。このため、かかる状況下でブレーキアシスト制御が開始されると、液圧流入経路の遮断されているホイルシリンダに供給されるべき制動液圧が、他のホイルシリンダに分配される事態が生ずる。液圧流入抑制手段は、かかる状況下で、他のホイルシリンダに過剰に制動液圧が導かれるのを防止する。

したがって、上述の発明によれば、何れかのホイルシリンダの液圧流入経路が遮断された状態でブレーキアシスト制御が開始されても、液圧流入経路の遮断されていないホイルシリンダに過剰に制動液圧が導かれるのを防止することができる。このため、本発明に係る制動力制御装置によれば、かかる状況下においても優れた制御性を維持することができる。

25 また、本発明の他の面によれば、車輪のスリップ状態に関する特性値が所定のしきい値を超える場合に、該車輪のホイルシリンダに連通する液圧流入経路を遮断した状態でホイルシリンダ圧を所定期間減圧する減圧制御を実行した後に、該ホイルシリンダについて所定の液圧制御を実行する制動液圧制御と、運転者によって緊急ブレーキ操作が実行された際に通常時に比して大きな制動液圧を発生させるブレーキアシスト制御とを実行する制動力制御装置において、

ホイルシリンダの液圧流入経路の導通状態を検出する導通検出手段と、

- 5 何れかのホイルシリンダの液圧流入経路が実質的に遮断された状態で前記ブレーキアシスト制御が開始された場合には、前記少なくとも他の一のホイルシリンダについて前記減圧制御が実行されることにより生ずる減圧傾向を、通常時に比して強める減圧傾向変更手段と、

を備える制動力制御装置が提供される。

- 10 上述の発明において、何れかのホイルシリンダについて液圧流入経路が遮断されている場合は、そのホイルシリンダへの制動液圧の流入が阻止される。このため、かかる状況下でブレーキアシスト制御が開始されると、他のホイルシリンダには、全てのホイルシリンダの液圧流入経路が導通状態である場合に比して急激な液圧上昇が生じ易い。本発明において、他の一のホイルシリンダのホイルシリンダ圧は、減圧制御が開始された後、通常時に比して大きく減圧される。このため、他の一のホイルシリンダのホイルシリンダ圧は、
15 何れかのホイルシリンダの液圧流入経路が遮断されている状況下であっても不必要に高圧にならない。

- 20 したがって、上述の発明によれば、何れかのホイルシリンダの液圧流入経路が遮断された状態でブレーキアシスト制御が開始されても、液圧流入経路の遮断されていないホイルシリンダに過剰に制動液圧が導かれるのを防止することができる。このため、本発明に係る制動力制御装置によれば、かかる状況下においても優れた制御性を維持することができる。

- 25 また、本発明による制動力制御装置は、

何れかのホイルシリンダの液圧流入経路が実質的に遮断された状態で前記ブレーキアシスト制御が開始された際に、少なくとも他の一のホイルシリンダについての前記しきい値を通常時に比して小さな値にするしきい値変更手段、

を備えることとしてもよい。

本発明において、何れかのホイルシリンダについて液圧流入経路が遮断された状態でブレーキアシスト制御が開始されると、他のホイルシリンダのホイルシリンダ圧は通常時に比して過渡に高圧となり易い。しきい値変更手段は、このような状況が生じた場合に、他のホイルシリンダについて減圧制御が開始され易いようにしきい値を変更する。このため、他のホイルシリンダのホイルシリンダ圧は、通常時に比して急上昇するにも関わらず、不必要に高い液圧にはならない。

したがって、上述の発明によれば、何れかのホイルシリンダの液圧流入経路が遮断された状態でブレーキアシスト制御が開始されても、液圧流入経路の遮断されていないホイルシリンダに過剰に制動液圧が導かれるのを防止することができる。このため、本発明に係る制動力制御装置によれば、かかる状況下においても優れた制御性を維持することができる。

また、本発明の他の面によれば、運転者によって緊急ブレーキ操作が行われた際に通常時に比して大きな制動油圧を発生させるブレーキアシスト制御と、各車輪の制動油圧を各車輪に過剰なスリップ率を発生させない圧力に制御するアンチロックブレーキ制御と、
20 を実行する制動力制御装置において、

ブレーキ操作量に応じた制動液圧を発生する操作液圧発生手段と、
ブレーキ操作量と無関係に所定の制動液圧を発生するアシスト圧発生手段と、

前記操作液圧発生手段および前記アシスト圧発生手段の双方に連
25 通する高圧通路と、

前記操作液圧発生手段と前記高圧通路とを遮断状態とし得る操作液圧カット機構と、

所定の低圧源に連通する低圧通路と、

各車輪のホイルシリンダと前記高圧通路との導通状態、および、

各車輪のホイルシリンダと前記低圧通路との導通状態を制御する導通状態制御機構と、

運転者によって緊急ブレーキ操作が行われた場合に、前記操作液圧カット機構を遮断状態とし、かつ、前記アシスト圧発生手段から

- 5 前記高圧通路に所定の制動液圧を供給させる B A 制御手段と、

前記導通状態制御機構を所定の制御パターンで制御することにより、各車輪に過剰なスリップ率が生じないように各車輪のホイルシリンダ圧を制御する A B S 制御手段と、

- アンチロックブレーキ制御が単独で実行されている場合に前記制
10 御パターンを通常パターンとし、アンチロックブレーキ制御とブレーキアシスト制御とが同時に実行されている場合に、前記制御パターンをホイルシリンダ圧の増圧量を抑制するための増圧量抑制パターンとする A B S 制御パターン選択手段と、

を備える制動力制御装置が提供される。

- 15 上述の発明において、運転者によって緊急ブレーキ操作が実行されることなく何れかの車輪に過剰なスリップ率が生じた場合は、アンチロックブレーキ制御（以下、A B S 制御）が単独で開始される。この場合、各車輪のホイルシリンダ圧は、ホイルシリンダと操作液圧発生手段とが導通状態とされた場合に増圧される。A B S 制御の
20 通常パターンは、かかる状況下でホイルシリンダ圧の増圧が図られた場合に、ホイルシリンダ圧に適当な増圧量が生ずるように設定されている。

- A B S 制御が開始される以前に運転者によって緊急ブレーキ操作が実行されると、ブレーキアシスト制御（以下、B A 制御と称す）
25 が開始される。B A 制御の実行中は、操作液圧カット機構によって操作液圧発生手段が高圧通路から切り離され、かつ、アシスト圧発生手段が高圧通路に所定の制動液圧を供給する。この場合、各車輪のホイルシリンダ圧は、操作液圧カット機構が閉じていることに起因して、アシスト圧発生手段を液圧源として速やかに増圧される。

5 B A 制御の開始に伴って各車輪のスリップ率が過大となった場合は、以後、B A 制御と A B S 制御とを同時に実行すること、すなわち、B A + A B S 制御を実行することが必要となる。A B S 制御の実行に先立って B A 制御が開始されている場合、A B S 制御は、高
10 圧通路にアシスト圧発生手段の発する高圧の制動液圧が導かれた状況下で実行される。本発明においては、かかる状況下では、増圧量抑制パターンにより A B S 制御が実行される。このため、高圧通路に通常時に比して高圧の制動液圧が導かれているにも関わらず、A B S 対象車輪のホイルシリンダ圧に過大な増圧量が生ずることがない。

したがって、上述の発明によれば、A B S 制御と B A 制御とが同時に実行されている場合に、A B S 対象車輪のホイルシリンダ圧に過剰な増圧量が付与されるのを、すなわち、A B S 制御のハンチングが生じ易い状況が形成されるのを防止することができる。

15 更に、本発明の他の面によれば、運転者によって緊急ブレーキ操作が行われた際に通常時に比して大きな制動油圧を発生させるブレーキアシスト制御と、各車輪の制動油圧を各車輪に過剰なスリップ率を発生させない圧力に制御するアンチロックブレーキ制御と、を実行する制動力制御装置において、

20 ブレーキ操作量に応じた制動液圧を発生する操作液圧発生手段と、ブレーキ操作量と無関係に所定の制動液圧を発生するアシスト圧発生手段と、

前記操作液圧発生手段および前記アシスト圧発生手段の双方に連通する高圧通路と、

25 前記操作液圧発生手段と前記高圧通路とを遮断状態とし得る操作液圧カット機構と、

所定の低圧源に連通する低圧通路と、

各車輪のホイルシリンダと前記高圧通路との導通状態、および、各車輪のホイルシリンダと前記低圧通路との導通状態を制御する導

通状態制御機構と、

運転者によって緊急ブレーキ操作が行われた場合に、前記操作液圧カット機構を遮断状態とし、かつ、前記アシスト圧発生手段から前記高圧通路に所定の制動液圧を供給させるBA制御手段と、

- 5 前記導通状態制御機構を所定の制御パターンで制御することにより、各車輪に過剰なスリップ率が生じないように各車輪のホイールシリンダ圧を制御するABS制御手段と、

- ブレーキアシスト制御とアンチロックブレーキ制御とが同時に実行されている場合に、アンチロックブレーキ制御の非対象車輪のホイールシリンダ圧の増圧勾配が抑制されるように、前記非対象車輪に対応して設けられている前記導通状態制御機構を制御するBA増圧勾配抑制手段と、
- 10

を備える制動力制御装置が提供される。

- 上述の発明において、運転者によって緊急ブレーキ操作が実行されると、BA制御が開始される。BA制御の実行中は、各車輪のホイールシリンダ圧がアシスト圧発生手段を液圧源として増圧される。アシスト圧発生手段には、高圧通路を介して連通する全てのホイールシリンダのホイールシリンダ圧 $P_{w/c}$ に、適当な増圧勾配を発生させるための能力が付与されている。
- 15

- 20 BA制御が開始された後、何れかの車輪について過剰なスリップ率が検出されると、BA+ABS制御が開始される。ABS対象車輪のホイールシリンダは、ABS制御によってその車輪のホイールシリンダ圧の増圧が要求される僅かな時間を除いて高圧通路から遮断される。このため、BA+ABS制御の実行中は、アシスト圧発生手段から吐出されるブレーキフルードのほぼ全量が、ABS非対象車輪のホイールシリンダに供給される。本発明においては、かかる状況が形成されると、ABS非対象車輪の増圧勾配が抑制されるように導通状態制御機構が制御される。このため、アシスト圧発生手段の能力が過剰であるにも関わらず、ABS非対象車輪のホイールシリン
- 25

ダ圧の増圧勾配が、B A制御が単独で実行されている場合と同様の適正な勾配に抑制される。

- したがって、上述の発明によれば、B A制御がA B S制御と同時に実行されている場合に、A B S非対象車輪のホイルシリンダ圧に
- 5 過剰な増圧勾配が生ずるのを防止することができる。

- また、本発明の他の面によれば、運転者によって緊急ブレーキ操作が行われた際に通常時に比して大きな制動油圧を発生させるブレーキアシスト制御と、各車輪の制動油圧を各車輪に過剰なスリップ率を発生させない圧力に制御するアンチロックブレーキ制御と、
- 10 を実行する制動力制御装置において、

- ブレーキ操作量に応じた制動液圧を発生する操作液圧発生手段と、第1低圧源および第2低圧源に連通する低圧通路と、
- 前記低圧通路から吸入したブレーキフルードを圧送することによりブレーキ操作量と無関係に所定の制動液圧を発生するアシスト圧
- 15 発生手段と、

- 前記操作液圧発生手段および前記アシスト圧発生手段の双方に連通する高圧通路と、
- 前記操作液圧発生手段と前記高圧通路とを遮断状態とし得る操作液圧カット機構と、
- 20 各車輪のホイルシリンダと前記高圧通路との導通状態、および、各車輪のホイルシリンダと前記低圧通路との導通状態を制御する導通状態制御機構と、

- 運転者によって緊急ブレーキ操作が行われた場合に、前記操作液圧カット機構を遮断状態とし、かつ、前記アシスト圧発生手段から
- 25 前記高圧通路に所定の制動液圧を供給させるB A制御手段と、

前記導通状態制御機構を所定の制御パターンで制御することにより、各車輪に過剰なスリップ率が生じないように各車輪のホイルシリンダ圧を制御するA B S制御手段と、

ブレーキアシスト制御とアンチロックブレーキ制御とが同時に実

行されている場合に、前記第 1 低圧源と前記アシスト圧発生手段とを遮断状態とする低圧源カット手段と、

を備える制動力制御装置が提供される。

上述の発明において、運転者によって緊急ブレーキ操作が実行されると、BA 制御が開始される。BA 制御の実行中は、各車輪のホイールシリンダ圧がアシスト圧発生手段を液圧源として増圧される。アシスト圧発生手段は、BA 制御が単独で実行されている場合は第 1 低圧源からブレーキフルードを吸入して高圧通路に制動液圧を供給する。この場合、高圧通路には多量のブレーキフルードが供給される。

BA 制御が開始された後、何れかの車輪について過剰なスリップ率が検出されると、BA + ABS 制御が開始される。ABS 制御は、ABS 対象車輪のホイールシリンダ圧が減圧されることにより、すなわち、ABS 対象車輪のホイールシリンダから低圧通路にブレーキフルードが放出されることにより開始される。このため、BA + ABS 制御が開始されると、即座に第 2 低圧源にブレーキフルードが流入する。

本発明において、BA + ABS 制御が開始されると、アシスト圧発生手段と第 1 低圧源とが遮断状態とされる。従って、アシスト圧発生手段が圧送できるブレーキフルードは、以後第 2 低圧源に貯留されているブレーキフルードだけに限定される。このため、BA + ABS 制御の実行中に高圧通路に不当に高圧の制動液圧が発生することはない。高圧通路に不当に高圧の制動液圧が発生しない状況下では、ABS 対象車輪のホイールシリンダ圧に過剰な増圧量が生ずることがなく、かつ、ABS 非対象車輪のホイールシリンダ圧 $P_{w/c}$ に過剰な増圧勾配が生ずることがないと共に、操作液圧発生手段にブレーキフルードが過剰に逆流することがない。

したがって、BA 制御と ABS 制御とが同時に実行される場合に、高圧通路に不当に高圧の制動液圧が発生するのを防止することがで

きる。

また、上術の発明による制動力制御装置において、

- ブレーキアシスト制御とアンチロックブレーキ制御とが同時に実行されており、かつ、アンチロックブレーキ制御の対象車輪において
5 ホイルシリンダ圧の減圧が図られている場合に、前記操作液圧カット機構を導通状態とする高圧通路開放手段を備えることとしてもよい。

- BA + ABS 制御が実行されている場合、アシスト圧発生手段は、上述の如く第2低圧源に貯留されているブレーキフルードのみを圧
10 送する。第2低圧源には、ABS 対象車輪のホイルシリンダ圧の減圧が図られる毎にブレーキフルードが放出される。このため、アシスト圧発生手段は、ABS 対象車輪でホイルシリンダ圧の減圧が図られる時期と同期して、高圧通路に多量のブレーキフルードを圧送する。

- 15 高圧通路は、アシスト圧発生手段によって多量のブレーキフルードが圧送される時期のみ操作液圧発生手段と導通状態とされる。高圧通路と操作液圧発生手段との導通状態が上記の如く制御されると、高圧通路内の制動液圧は、操作液圧発生手段が発する制動液圧に比して高い適当な圧力に制御される。このため、本発明によれば、A
20 BS 対象車輪に制御上のハンチングを発生させることなく、ABS 非対象車輪のホイルシリンダ圧を適当な増圧勾配で増圧することができる。

- したがって、BA 制御とABS 制御とが同時に実行される場合に、ABS 対象車輪に制御上のハンチングが生ずるのを防止することができる
25 できると共に、ABS 非対象車輪のホイルシリンダ圧に過剰な増圧勾配が生ずるのを防止することができる。

また、本発明の更に他の面によれば、運転者によって緊急ブレーキ操作が行われた際に通常時に比して大きな制動油圧を発生させるブレーキアシスト制御と、各車輪の制動油圧を各車輪に過剰なス

リップ率を発生させない圧力に制御するアンチロックブレーキ制御と、を実行する制動力制御装置において、

ブレーキ操作量に応じた制動液圧を発生する操作液圧発生手段と、

ブレーキ操作量と無関係に所定の制動液圧を発生するアシスト圧

5 発生手段と、

前記操作液圧発生手段および前記アシスト圧発生手段の双方に連通する高圧通路と、

前記操作液圧発生手段と前記高圧通路とを遮断状態とし得る操作液圧カット機構と、

10 所定の低圧源に連通する低圧通路と、

各車輪のホイールシリンダと前記高圧通路との導通状態、および、各車輪のホイールシリンダと前記低圧通路との導通状態を制御する導通状態制御機構と、

運転者によって緊急ブレーキ操作が行われた場合に、前記操作液
15 圧カット機構を遮断状態とし、かつ、前記アシスト圧発生手段から前記高圧通路に所定の制動液圧を供給させるBA制御手段と、

前記導通状態制御機構を所定の制御パターンで制御することにより、各車輪に過剰なスリップ率が生じないように各車輪のホイールシリンダ圧を制御するABS制御手段と、

20 ブレーキアシスト制御とアンチロックブレーキ制御とが同時に実行されている場合に、前記操作液圧カット機構を導通状態とする高圧通路開放手段と、

を備える制動力制御装置が提供される。

上述の発明において、BA+ABS制御の実行中は、ABS対象
25 車輪のホイールシリンダがほぼ高圧通路と切り離された状態とされることに起因して、アシスト圧発生手段の吐出能力が過剰となる。この際、本発明においては、高圧通路と操作液圧発生手段とが導通状態とされる。高圧通路と操作液圧発生手段とが導通状態とされると、アシスト圧発生手段によって吐出されるブレーキフルードが操作液

圧発生手段に流入することが可能となる。従って、アシスト圧発生手段の吐出能力が過剰であっても、高圧通路に不当に高圧の制動液圧が生ずることがない。

- したがって、B A制御とA B S制御とが同時に実行される場合に、
5 高圧通路に不当に高圧の制動液圧が発生するのを防止することができる。

本発明の他の目的、特徴及び利点は添付の図面を参照しながら以下の詳細な説明を読むことにより一層明瞭となるであろう。

10 図面の簡単な説明

図1は、本発明の第1乃至第3実施例による制動力制御装置のシステム構成図である。

図2は、図1に示す制動力制御装置がA B S制御を実行する際に実現されるホイールシリンダ圧 $P_{w/c}$ の変化を示すグラフである。

- 15 図3は、図1に示す制動力制御装置が備えるホイールシリンダに液圧源を接続した場合に実現される昇圧特性を示すグラフである。

図4は、図1に示す制動力制御装置が備える後輪のホイールシリンダが種々の状況下で示す増圧勾配を表すグラフである。

- 20 図5は、図1に示す制動力制御装置において実現されるホイールシリンダ圧のオーバーシュートを説明するためのグラフである。

図6は、本発明の第1実施例による制動力制御装置において実行される制御ルーチンのフローチャートである。

- 25 図7は、本発明の第1実施例による制動力制御装置において図6に示す制御ルーチンが実行された際に実現されるホイールシリンダ圧の変化を示すグラフである。

図8は、本発明の第2実施例による制動力制御装置において実行される制御ルーチンのフローチャートである。

図9は、本発明の第2実施例による制動力制御装置において図8に示す制御ルーチンが実行された際に実現されるホイールシリンダ圧

の変化を示すグラフである。

図 10 は、B A 制御の実行・停止に伴う液圧源および増圧特性の変化を示す図である。

図 11 は、本発明の第 3 実施例による制動力制御装置において実行される制御ルーチンのフローチャートである。

図 12 は、本発明の第 4 実施例による制動力制御装置の通常ブレーキ状態および A B S 作動状態を示すシステム構成図である。

図 13 は、図 12 に示す制動力制御装置において B A 制御中に実現されるアシスト圧増圧状態を示す図である。

10 図 14 は、図 12 に示す制動力制御装置において B A 制御中に実現されるアシスト圧保持状態を示す図である。

図 15 は、図 12 に示す制動力制御装置において B A 制御中または B A + A B S 制御中に実現されるアシスト圧減圧状態を示す図である。

15 図 16 は、図 12 に示す制動力制御装置において B A + A B S 制御中に実現されるアシスト圧増圧状態を示す図である。

図 17 は、図 12 に示す制動力制御装置において B A + A B S 制御中に実現されるアシスト圧保持状態を示す図である。

図 18 は、図 12 に示す制動力制御装置においてリザーバカットソレノイドの状態を制御するために実行される制御ルーチンのフローチャートである。

図 19 は、図 12 に示す制動力制御装置において保持ソレノイドおよび減圧ソレノイドの制御手法を選択するために実行される制御ルーチンのフローチャートである。

25 図 20 は、図 12 に示す制動力制御装置において A B S 制御を実現するために実行される制御ルーチンのフローチャートである。

図 21 は、図 12 に示す制動力制御装置においてマスタカットソレノイドの状態を制御するために実行される制御ルーチンのフローチャートである。

図 2 2 は、本発明の第 5 実施例による制動力制御装置の通常ブレーキ状態および A B S 作動状態を示すシステム構成図である。

図 2 3 は、図 2 2 に示す制動力制御装置において B A 制御中に実現されるアシスト圧増圧状態を示す図である。

5 図 2 4 は、図 2 2 に示す制動力制御装置において B A 制御中に実現されるアシスト圧保持状態を示す図である。

図 2 5 は、図 2 2 に示す制動力制御装置において B A 制御中または B A + A B S 制御中に実現されるアシスト圧減圧状態を示す図である。

10 図 2 6 は、図 2 2 に示す制動力制御装置において B A + A B S 制御中に実現されるアシスト圧増圧状態を示す図である。

図 2 7 は、図 2 2 に示す制動力制御装置において B A + A B S 制御中に実現されるアシスト圧保持状態を示す図である。

15 発明を実施するための最良の形態

図 1 は、本発明の第 1 実施例による制動力制御装置のシステム構成図である。図 1 に示す制動力制御装置は、電子制御ユニット 2 0 (以下、E C U 2 0 と称す) により制御されている。制動力制御装置は、ポンプ 2 1 を備えている。ポンプ 2 1 は、その動力源として
20 モータ 2 2 を備えている。ポンプ 2 1 の吸入口 2 1 a はリザーバタンク 2 3 に連通している。また、ポンプ 2 1 の吐出口 2 1 b には、逆止弁 2 4 を介してアキュムレータ 2 5 が連通している。ポンプ 2 1 は、アキュムレータ 2 5 内に、常に所定の液圧が蓄圧されるように、リザーバタンク 2 3 内のブレーキフルードを、その吐出口 2 1
25 b から圧送する。

アキュムレータ 2 5 は、高压通路 2 6 を介してレギュレータ 2 7 の高压ポート 2 7 a、およびレギュレータ切り換えソレノイド 2 8 (以下、S T R 2 8 と称す) に連通している。レギュレータ 2 7 は、低压通路 2 9 を介してリザーバタンク 2 3 に連通する低压ポート 2

7 b と、制御液圧通路 3 0 を介して S T R 2 8 に連通する制御液圧ポート 2 7 c を備えている。S T R 2 8 は、制御液圧通路 3 0 および高圧通路 2 6 の一方を選択的に導通状態とする 2 位置の電磁弁であり、常態では、制御液圧通路 3 0 を導通状態とし、かつ、高圧通路 2 6 を遮断状態とする。ここで、2 位置の電磁弁とは 2 つの状態に切り替えることができる電磁弁を意味する。

レギュレータ 2 7 には、ブレーキペダル 3 1 が連結されていると共に、マスタシリンダ 3 2 が固定されている。レギュレータ 2 7 は、その内部に液圧室を備えている。液圧室は、常に制御液圧ポート 2 7 c に連通されていると共に、ブレーキペダル 3 1 の操作状態に応じて、選択的に高圧ポート 2 7 a または低圧ポート 2 7 b に連通される。レギュレータ 2 7 は、液圧室の内圧が、ブレーキペダル 3 1 に作用するブレーキ踏力 F_P に応じた液圧に調整されるように構成されている。このため、レギュレータ 2 7 の制御液圧ポート 2 7 c には、常に、ブレーキ踏力 F_P に応じた液圧が表れる。以下、この液圧をレギュレータ圧 P_{RE} と称す。

ブレーキペダル 3 1 に作用するブレーキ踏力 F_P は、レギュレータ 2 7 を介して機械的にマスタシリンダ 3 2 に伝達される。また、マスタシリンダ 3 2 には、レギュレータ 2 7 の液圧室の液圧に応じた、すなわちレギュレータ圧 P_{RE} に応じた力が伝達される。以下、この力をブレーキアシスト力 F_A と称す。従って、ブレーキペダル 3 1 が踏み込まれると、マスタシリンダ 3 2 には、ブレーキ踏力 F_P とブレーキアシスト力 F_A との合力が伝達される。

マスタシリンダ 3 2 は、その内部に第 1 液圧室 3 2 a と第 2 液圧室 3 2 b とを備えている。第 1 液圧室 3 2 a および第 2 液圧室 3 2 b には、ブレーキ踏力 F_P とブレーキアシスト力 F_A との合力に応じたマスタシリンダ圧 $P_{M/c}$ が発生する。第 1 液圧室 3 2 a に発生するマスタシリンダ圧 $P_{M/c}$ および第 2 液圧室 3 2 b に発生するマスタシリンダ圧 $P_{M/c}$ は、共にプロポーショニングバルブ 3 4 (以

下、Pバルブ34と称す)に連通している。

Pバルブ34には、第1液圧通路36と第2液圧通路38とが連通している。Pバルブ34は、マスタシリンダ圧 $P_{M/c}$ が所定値に満たない領域では、第1液圧通路36および第2液圧通路38に対して、マスタシリンダ圧 $P_{M/c}$ をそのまま供給する。また、Pバルブ34は、マスタシリンダ圧 $P_{M/c}$ が所定値を超える領域では、第1液圧通路36に対してマスタシリンダ圧 $P_{M/c}$ をそのまま供給すると共に、第2液圧通路38に対してマスタシリンダ圧 $P_{M/c}$ を所定の比率で減圧した液圧を供給する。

第2液圧通路38には、マスタシリンダ圧 $P_{M/c}$ に比例した電気信号を出力する液圧センサ40が加設されている。液圧センサ40の出力信号はECU20に供給されている。ECU20は、液圧センサ40の出力信号に基づいて、マスタシリンダ32に生じているマスタシリンダ圧 $P_{M/c}$ を検出する。

上述したSTR28には、第3液圧通路42が連通している。第3液圧通路42は、STR28の状態に応じて、制御液圧通路30または高压通路26の一方と連通状態とされる。本実施例において、左右前輪FL, FRに配設されるホイルシリンダ44FL, 44FRには、Pバルブ34に連通する第1液圧通路36、または、STR28に連通する第3液圧通路42から制動液圧が供給される。また、左右後輪RL, RRに配設されるホイルシリンダ44RL, 44RRには、Pバルブ34に連通する第2液圧通路38、または、STR28に連通する第3液圧通路42から制動液圧が供給される。

第1液圧通路36には、第1アシストソレノイド46(以下、SA-146と称す)、および第2アシストソレノイド48(以下、SA-248と称す)が連通している。一方、第3液圧通路42には、右前輪保持ソレノイド50(以下、SFRH50と称す)、左前輪保持ソレノイド52(以下、SFLH52と称す)、および第3アシストソレノイド54(以下、SA-354と称す)が連通している

ここで、本明細書においてソレノイドとはソレノイドバルブを意味する。

SFRH50は、常態では開弁状態を維持する2位置の電磁開閉弁である。SFRH50は、調圧用液圧通路56を介して、SA-1
5 46および右前輪減圧ソレノイド58（以下、SFRR58と称す）に連通している。第3液圧通路42と調圧用液圧通路56との間には、調圧用液圧通路56側から第3通路42側へ向かう流体の流れのみを許容する逆止弁60が並設されている。

SA-146は、第1液圧通路36および調圧用液圧通路56の一方を選択的にホイルシリンダ44FRに導通させる2位置の電磁弁
10 であり、常態（オフ状態）では、第1液圧通路36とホイルシリンダ44FRとを導通状態とする。一方、SFRR58は、調圧用液圧通路56とリザーバタンク23とを導通状態または遮断状態とする2位置の電磁開閉弁である。SFRR58は、常態（オフ状態）
15 では調圧用液圧通路56とリザーバタンク23とを遮断状態とする。

SFLH52は、常態では開弁状態を維持する2位置の電磁開閉弁である。SFLH52は、調圧用液圧通路62を介して、SA-2
48および左前輪減圧ソレノイド64（以下、SFLR64と称す）に連通している。第3液圧通路42と調圧用液圧通路62との
20 間には、調圧用液圧通路62側から第3通路42側へ向かう流体の流れのみを許容する逆止弁66が並設されている。

SA-248は、第1液圧通路36および調圧用液圧通路62の一方を、選択的にホイルシリンダ44FLに導通させる2位置の電磁
25 弁であり、常態（オフ状態）では、第1液圧通路36とホイルシリンダ44FLとを導通状態とする。一方、SFLR64は、調圧用液圧通路62とリザーバタンク23とを導通状態または遮断状態とする2位置の電磁開閉弁である。SFLR64は、常態（オフ状態）では調圧用液圧通路62とリザーバタンク23とを遮断状態とする。

第2液圧通路38は、上述したSA-354に連通している。SA-354の下流側には、右後輪RRのオイルシリンダ44RRに対応して設けられた右後輪保持ソレノイド68（以下、SRRH68と称す）、および、左後輪RLのオイルシリンダ44RLに対応して
5 設けられた左後輪保持ソレノイド70（以下、SRLR70）が連通している。SA-354は、第2液圧通路38および第3液圧通路42の一方を、選択的にSRRH68およびSRLR70に連通させる2位置の電磁弁であり、常態（オフ状態）では、第2液圧通路38とSRRH68およびSRLR70とを連通状態とする。

10 SRRH68の下流側には、調圧用液圧通路72を介して、オイルシリンダ44RR、および、右後輪減圧ソレノイド74（以下、SRRR74と称す）が連通している。SRRR74は、調圧用液圧通路72とリザーバタンク23とを導通状態または遮断状態とする2位置の電磁開閉弁であり、常態（オフ状態）では調圧用液圧通路72とリザーバタンク23とを遮断状態とする。また、SA-354と調圧用液圧通路72との間には、調圧用液圧通路72側からSA-354側へ向かう流体の流れのみを許容する逆止弁76が並設されている。
15

同様に、SRLH70の下流側には、調圧用液圧通路78を介して、
20 ホイルシリンダ44RL、および、左後輪減圧ソレノイド80（以下、SRLR80と称す）が連通している。SRLR80は、調圧用液圧通路78とリザーバタンク23とを導通状態または遮断状態とする2位置の電磁開閉弁であり、常態（オフ状態）では調圧用液圧通路78とリザーバタンク23とを遮断状態とする。また、
25 SA-354と調圧用液圧通路78との間には、調圧用液圧通路78側からSA-354側へ向かう流体の流れのみを許容する逆止弁82が並設されている。

本実施例のシステムにおいて、ブレーキペダル31の近傍には、ブレーキスイッチ84が配設されている。ブレーキスイッチ84は、

ブレーキペダル 31 が踏み込まれている場合にオン出力を発するスイッチである。ブレーキスイッチ 84 の出力信号は ECU 20 に供給されている。ECU 20 は、ブレーキスイッチ 84 の出力信号に基づいて、運転者によって制動操作がなされているか否かを判別する。

また、本実施例のシステムにおいて、左右前輪 FL, FR および左右後輪 RL, RR の近傍には、それぞれ各車輪が所定回転角回転する毎にパルス信号を発する車輪速センサ 86 FL, 86 FR, 86 RL, 86 RR (以下、これらを総称する場合は符号 86.. を付して表す) が配設されている。車輪速センサ 86.. の出力信号は ECU 20 に供給されている。ECU 20 は、車輪速センサ 86.. の出力信号に基づいて、各車輪 FL, FR, RL, RR の回転速度、すなわち、各車輪 FL, FR, RL, RR の車輪速度を検出する。

ECU 20 は、液圧センサ 40、車輪速センサ 86..、および、ブレーキスイッチ 84 の出力信号に基づいて、上述した STR28、SA-146、SA-248、SA-354、SFRH50、SFLH52、SFRR58、SFLR64、SRRH68、SRLH70、SRRR74、および、SRLR80 に対して適宜駆動信号を供給する。

次に、本実施例の制動力制御装置の動作を説明する。本実施例の制動力制御装置は、車両状態が安定している場合は、ブレーキペダル 31 に作用するブレーキ踏力 F_p に応じた制動力を発生させる通常制御を実行する。通常制御は、図 1 に示す如く、STR28、SA-146、SA-248、SA-354、SFRH50、SFLH52、SFRR58、SFLR64、SRRH68、SRLH70、SRRR74、および、SRLR80 を全てオフ状態とすることで実現される。

すなわち、図 1 に示す状態においては、ホイルシリンダ 44 FR および 44 FL は第 1 液圧通路 36 に、また、ホイルシリンダ 44

RRおよび44RLは第2液圧通路38にそれぞれ連通される。この場合、ブレーキフルードは、マスタシリンダ32とホイールシリンダ44FR, 44FL, 44RL, 44RR（以下、これらを総称する場合は符号44..を付して表す）との間で授受されることとなり、各車輪FL, FR, RL, RRにおいて、ブレーキ踏力 F_p に応じた制動力が発生される。

本実施例において、何れかの車輪についてロック状態へ移行する可能性があることが検出されると、その車輪についてアンチロックブレーキ制御（以後、ABS制御と称す）の実行条件が成立したと判定され、以後、ABS制御が開始される。ECU20は、車輪速センサ86..の出力信号に基づいて各車輪の車輪速度 V_{wFL} , V_{wFR} , V_{wRL} , V_{wRR} （以下、これらを総称する場合は符号 $V_{w..}$ を付して表す）を演算し、それらの車輪速度 $V_{w..}$ に基づいて、公知の手法により車体速度の推定値 V_{so} （以下、推定車体速度 V_{so} と称す）を演算する。そして、車両が制動状態にある場合に、次式に従って個々の車輪のスリップ率 S を演算し、 S が所定値を超えている場合に、その車輪がロック状態に移行する可能性があると判断する。

$$S = (V_{so} - V_{w..}) \cdot 100 / V_{so} \quad \dots (1)$$

ECU20は、右前輪FRについてABS制御の実行条件が成立すると判断した場合はSA-146に対して駆動信号を出力する。また、ECU20は、左前輪FRについてABS制御の実行条件が成立すると判断した場合はSA-248に対して駆動信号を出力する。そして、ECU20は、左右後輪RL, RRの何れかについてABS制御の実行条件が成立すると判断した場合はSA-354に対して駆動信号を出力する。

SA-146がオン状態とされると、ホイールシリンダ44FRが、第1液圧通路36から遮断されて調圧用液圧通路56に連通される。また、SA-248がオン状態とされると、ホイールシリンダ44FL

が、第 1 液圧通路 3 6 から遮断されて調圧用液圧通路 6 2 に連通される。更に、 $SA_{-3}54$ がオン状態とされると、 $SRRH68$ および $SRLH70$ が第 2 液圧通路 3 8 から遮断されて第 3 液圧通路 4 2 に連通される。

- 5 上記の如く $SA_{-1}46$ 、 $SA_{-2}48$ および $SA_{-3}54$ がオン状態とされると、ホイルシリンダ 4 4 .. が、対応する保持ソレノイド $SFRH50$ 、 $SFLH52$ 、 $SRRH68$ 、 $SRLH70$ （以下、これらを総称する場合は、保持ソレノイド $S_{..}H$ と称す）、および、対応する減圧ソレノイド $SFRR58$ 、 $SFLR64$ 、 $SRRR7$
 10 4、 $SRLR80$ （以下、これらを総称する場合は、減圧ソレノイド $S_{..}R$ と称す）に連通し、かつ、保持ソレノイド $S_{..}H$ に、第 3 液圧通路 4 2 および $STR28$ を介して、レギュレータ圧 P_{RE} が導かれる状態が形成される。

- 上記の状況下で、保持ソレノイド $S_{..}H$ が開弁状態とされ、かつ、
 15 減圧ソレノイド $S_{..}R$ が閉弁状態とされると、対応するホイルシリンダ 4 4 .. のホイルシリンダ圧 P_{wc} が、レギュレータ圧 P_{RE} を上限値として増圧される。以下、この状態を①増圧モードと称す。また、上記の状況下で保持ソレノイド $S_{..}H$ が閉弁状態とされ、かつ、減圧ソレノイド $S_{..}R$ が閉弁状態とされると、対応するホイルシリン
 20 ンダ 4 4 .. のホイルシリンダ圧 P_{wc} が増減されることなく保持される。以下、この状態を②保持モードと称す。更に、上記の状況下で保持ソレノイド $S_{..}H$ が閉弁状態とされ、かつ、減圧ソレノイド $S_{..}R$ が開弁状態とされると、対応するホイルシリンダ 4 4 .. のホイルシリンダ圧 P_{wc} が減圧される。以下、この状態を③減圧モード
 25 と称す。

ECU 2 0 は、制動時における各車輪のスリップ率 S が適当な値に収まるように、すなわち、各車輪がロック状態に移行しないように、適宜上述した①増圧モード、②保持モードおよび③減圧モードを実現する。図 2 は、ECU 2 0 がこれらのモードを組み合わせて

A B S制御を実行する際に実現されるホイルシリンダ圧 $P_{w/c}$ の経時的変化を示す。

図2は、時刻 t_0 にブレーキ操作が開始され、時刻 t_1 にA B S制御の実行条件が成立した場合を示す。時刻 t_0 の後、ホイルシリンダ圧 $P_{w/c}$ が上昇し、時刻 t_1 に車輪のスリップ率 S が所定値に達すると、A B S制御が開始される。尚、以下の記載においては、車輪のスリップ率 S が所定値に到達した際のホイルシリンダ圧 $P_{w/c}$ をA B S作動油圧と称す。

A B S制御の実行条件が成立すると、先ずホイルシリンダ圧 $P_{w/c}$ をA B S作動油圧から減圧すべく②減圧モードが実現される。A B S制御の実行条件が成立した後に減圧モードが維持される時間（以下、初回減圧時間と称す）はA B S制御の実行条件が成立した際の車輪のスリップ状態に応じて決定される。具体的には、車輪のスリップ率が緩やかに増加している場合は、初回減圧時間が比較的短く設定され、一方、車輪のスリップ率が急激に増加している場合は、初回減圧時間が比較的長時間に設定される。

図2は、初回の減圧モードが時刻 t_2 まで維持された場合を示す。初回の減圧モードを維持すべき時間が経過すると、次に③保持モードが実現される。その後、保持モードを維持すべき所定時間が経過すると、時刻 t_3 に①増圧モードが開始される。そして、増圧モードが所定時間維持された後、時刻 t_4 に緩増圧モード（以下、④を付して表す）が開始される。緩増圧モードは、①増圧モードと③保持モードとが交互に実行されることで実現されるモードである。以後、ホイルシリンダ圧 $P_{w/c}$ が再びA B S作動圧に到達すると、再び上述した一連の制御、すなわち、②減圧モード→③保持モード→①増圧モード→④緩増圧モードを順次実現する処理が実行される。

A B S制御の実行中、②減圧モードが実行される期間中、③保持モードが実行される期間中、および、④緩増圧が実行される期間の殆どは、A B S制御の対象とされているホイルシリンダ44..が、

対応する保持ソレノイド $S_{..H}$ によって液圧源（マスタシリンダ32およびレギュレータ27）から遮断された状態となる。このように、ABS制御の対象であるホイルシリンダは、ABS制御の実行期間中、ほぼ液圧源から切り離された状態とされる。

- 5 ABS制御の実行中に、運転者によってブレーキペダル31の踏み込みが解除された後は、速やかにホイルシリンダ圧 $P_{w/c}$ が減圧される必要がある。本実施例のシステムにおいて、各ホイルシリンダ44..に対応する油圧経路中には、ホイルシリンダ44..側から第3液圧通路42側へ向かう流体の流れを許容する逆止弁60, 6
- 10 6, 76, 82が配設されている。このため、本実施例のシステムによれば、ブレーキペダル31の踏み込みが解除された後に、速やかに全てのホイルシリンダ44..のホイルシリンダ圧 $P_{w/c}$ を減圧させることができる。

- 本実施例のシステムにおいてABS制御が実行されている場合、
- 15 ホイルシリンダ圧 $P_{w/c}$ は、ホイルシリンダ44..に対してレギュレータ27からブレーキフルードが供給されることにより、すなわち、ホイルシリンダ44..に対してポンプ21からブレーキフルードが供給されることにより増圧される。また、ホイルシリンダ圧 $P_{w/c}$ は、ホイルシリンダ44..内のブレーキフルードがリザーバタンク23に流出されることにより減圧される。ホイルシリンダ圧 $P_{w/c}$ の増圧が、マスタシリンダ32を液圧源として行われるとすれば、増圧モードと減圧モードとが繰り返し行われた場合に、マスタシリンダ32内のブレーキフルードが徐々に減少し、いわゆるマスタシリンダの床付きが生ずる場合がある。

- 25 これに対して、本実施例のシステムの如く、ポンプ21を液圧源としてホイルシリンダ圧 $P_{w/c}$ の昇圧を図ることとすれば、かかる床付きを防止することができる。このため、本実施例のシステムによれば、長期間にわたってABS制御が続行される場合においても、安定した作動状態を維持することができる。

ところで、本実施例のシステムにおいて、ABS制御は、何れかの車輪について、ロック状態に移行する可能性が検出された場合に開始される。従って、ABS制御が開始させるためには、その前提として、何れかの車輪に大きなスリップ率 S が生ずる程度の制動操作がなされる必要がある。

車両の運転者が上級者である場合は、緊急ブレーキが必要とされる状況が生じた後、速やかにブレーキ踏力 F_p を急上昇させ、かつ、大きなブレーキ踏力 F_p を長期間にわたって維持することができる。ブレーキペダル31に対してかかるブレーキ踏力 F_p が作用すれば、マスタシリンダ32から各ホイールシリンダ44..に対して十分に高圧の制動液圧を供給することができ、ABS制御を開始させることができる。

しかしながら、車両の運転者が初級者である場合は、緊急ブレーキが必要とされる状況が生じた後、ブレーキ踏力 F_p が十分に大きな値にまで上昇されない場合がある。ブレーキペダル31に作用するブレーキ踏力 F_p が、緊急ブレーキが必要となった後十分に上昇されない場合には、各ホイールシリンダ44..のホイールシリンダ圧 $P_{w/c}$ が十分に昇圧されず、ABS制御が開始されない可能性がある。

このように、車両の運転者が初級者であると、車両が優れた制動能力を有しているにも関わらず、緊急制動操作時でさえ、その能力が十分に発揮されない場合がある。そこで、本実施例のシステムにおいては、ブレーキペダル31が緊急ブレーキを意図して操作され、かつ、ブレーキ踏力 F_p が十分に上昇されない場合に、強制的にホイールシリンダ圧 $P_{w/c}$ を昇圧させる制御を実行することとしている。以下、この制御をブレーキアシスト制御(BA制御)と称す。

本実施例のシステムにおいて、ブレーキペダル31にブレーキ踏力 F_p が付与されると、マスタシリンダ32には、ブレーキ踏力 F_p に応じたマスタシリンダ圧 $P_{m/c}$ が発生する。通常の制動操作が行われた場合は、緊急ブレーキを意図する制動操作が行われた場合

に比してマスタシリンダ圧 $P_{m/c}$ が緩やかに変化する。また、通常の制動操作に伴って生ずるマスタシリンダ圧 $P_{m/c}$ は、緊急ブレーキを意図する制動操作に伴って生ずるマスタシリンダ圧 $P_{m/c}$ に比してその収束値が低圧である。

- 5 このため、制動操作が開始された後、液圧センサ 40 に検出されるマスタシリンダ圧 $P_{m/c}$ が、所定値を超える変化率で、かつ、十分に大きな値にまで上昇された場合は、緊急ブレーキを意図する制動操作が行われたと判断することができる。また、制動操作が開始された後、マスタシリンダ圧 $P_{m/c}$ が所定値に比して小さな変化率
- 10 を示す場合、および、マスタシリンダ圧 $P_{m/c}$ の収束値が所定値に到達しない場合は、通常ブレーキを意図する制動操作が行われたと判断することができる。

- 本実施例においては、液圧センサ 40 の検出値であるマスタシリンダ圧 $P_{m/c}$ （以下、その値を検出値 $S P_{m/c}$ と称す）、および、
- 15 その変化率 $\Delta S P_{m/c}$ が所定の緊急ブレーキ条件を満たし、かつ、検出値 $S P_{m/c}$ が十分に昇圧されない場合に（以下、これらの条件を総称して B A 制御の実行条件と称す） B A 制御の実行を開始することとしている。

- 以下、 B A 制御の実行に伴う本実施例のシステムの動作について
- 20 説明する。運転者によって緊急ブレーキ条件を満たす制動操作が実行されると、 E C U 20 において B A 制御の実行条件が成立したと判断される。 E C U 20 は、 B A 制御の実行条件が成立すると判断した後、アキュムレータ 25 を液圧源とする方が、マスタシリンダ圧 $P_{m/c}$ が液圧源とするよりもホイールシリンダ圧 $P_{w/c}$ を急昇圧する
- 25 うえで有利となる状況が形成されているか否かを判断する。その結果、アキュムレータ 25 を液圧源とする方が有利な状況が形成されていると、 E C U 20 において B A 制御の開始タイミングが到来したと判断される。

 E C U 20 は、 B A 制御の開始タイミングが到来したと判断する

と、STR 28、SA-₁46、SA-₂48およびSA-₃54に対して駆動信号を出力する。上記の駆動信号を受けてSTR 28がオン状態となると、第3液圧通路42と高压通路26とが直結状態となる。この場合、第3液圧通路42には、アキュムレータ圧 P_{acc} が導かれる。また、上記の駆動信号を受けてSA-₁46およびSA-₂48がオン状態となると、ホイルシリンダ44FRおよび44FLが、それぞれ調圧用液圧通路56および62に連通される。更に、上記の駆動信号を受けてSA-₃54がオン状態となると、SRRH 68およびSRLH 70の上流側が第3液圧通路42に連通される。この場合、全てのホイルシリンダ44..が、それぞれの保持ソレノイドS..H、および、それぞれの減圧ソレノイドS..Rに連通し、かつ、全ての保持ソレノイドS..Hの上流に、アキュムレータ圧 P_{acc} が導かれる状態が形成される。

BA制御の開始タイミングが到来したと判断される時点で、ABS制御等の他の制動力制御が実行されていない場合は、その時点で全ての保持ソレノイドS..H、および、全ての減圧ソレノイドS..Rがオフ状態に維持されている。従って、上記の如く、保持ソレノイドS..Hの上流にアキュムレータ圧 P_{acc} が導かれると、その液圧はそのままホイルシリンダ44..に供給される。その結果、全てのホイルシリンダ44..のホイルシリンダ圧 $P_{w/c}$ は、アキュムレータ圧 P_{acc} に向けて昇圧される。

このように、本実施例のシステムによれば、緊急制動操作が実行された場合に、ブレーキ踏力 F_p の大きさとは無関係に、全てのホイルシリンダ44..のホイルシリンダ圧 $P_{w/c}$ を速やかに急昇圧させることができる。従って、本実施例のシステムによれば、運転者が初級者であっても、緊急ブレーキが必要とされる状況が生じた後に、速やかに大きな制動力を発生させることができる。

緊急制動操作が行われることにより、上記の如くBA制御が開始された場合、ブレーキペダル31の踏み込みが解除された時点で、

BA制御を終了させる必要がある。本実施例のシステムにおいて、BA制御が実行されている間は、上述の如くSTR28、SA-146、SA-248、およびSA-354がオン状態に維持される。STR28、SA-146、SA-248、およびSA-354がオン状態である場合、レギュレータ27内部の液圧室、およびマスタシリンダ32が備える第1および第2液圧室32a、32bが、実質的には何れも閉空間となる。

かかる状況下では、マスタシリンダ圧 $P_{m/c}$ は、ブレーキ踏力 F_p に応じた値となる。従って、ECU20は、液圧センサ40により検出されるマスタシリンダ圧 $P_{m/c}$ の出力信号を監視することにより、容易にブレーキペダル31の踏み込みが解除されたか否かを判断することができる。ブレーキペダル31の踏み込みの解除を検出すると、ECU20は、STR28、SA-146、SA-248、およびSA-354に対する駆動信号の供給を停止して、通常制御の
15 実行状態を実現する。このように、本実施例のシステムによれば、制動操作の終了と共に確実にBA制御を終了させることができる。

ホイールシリンダ44..に対して、上記の如くアキュムレータ圧 P_{acc} が供給され始めると、その後、各車輪FL、FR、RL、RRのスリップ率Sが急激に増大され、やがてABS制御の実行条件が
20 成立する。ABS制御の実行条件が成立すると、ECU20は、全ての車輪のスリップ率Sが適当な値に収まるように、すなわち、各車輪がロック状態に移行しないように、適宜上述した①増圧モード、②保持モード、および、③減圧モードを組み合わせてなるABS制御を実行する。

25 尚、BA制御が開始された後にABS制御が実行される場合、ホイールシリンダ圧 $P_{w/c}$ は、ポンプ21およびアキュムレータ25からホイールシリンダ44..にブレーキフルードが供給されることにより増圧されると共に、ホイールシリンダ44..内のブレーキフルードがリザーバタンク23に流出することにより減圧される。従って、

増圧モードと減圧モードとが繰り返し行われても、いわゆるマスタシリンダ 3 2 の床付きが生ずることはない。

次に、B A 制御が実行されることにより実現されるホイルシリンダ圧 $P_{w/c}$ の増圧勾配について説明する。図 3 は、時刻 t_s に圧力 P_0 を蓄える液圧源をホイルシリンダ 4 4 .. に導通させた場合に実現されるホイルシリンダ圧 $P_{w/c}$ の昇圧曲線を示す。図 3 に示す如く、ホイルシリンダ 4 4 .. のホイルシリンダ圧 $P_{w/c}$ は、時刻 t_s の後に急上昇した後、上昇率を緩めながら圧力 P_0 に収束する。この際、急上昇区間におけるホイルシリンダ圧 $P_{w/c}$ の増圧勾配 dP/dt は、圧力 P_0 が高压であるほど大きくなると共に、液圧源の液圧貯留量が高いほど、すなわち、液圧源の液圧供給能力が高いほど大きくなる。

図 4 は、左右後輪 R L, R R のホイルシリンダ 4 4 R L, 4 4 R R で実現されるホイルシリンダ圧 $P_{w/c}$ の増圧勾配 dB/dt を示す。図 4 中に破線で示される折れ線は通常制御時に急ブレーキ操作が行われた際に実現される増圧勾配 dB/dt を表している。また、図 4 中に実線で示される折れ線、一点鎖線で示される折れ線、および、二点鎖線で示される折れ線は、それぞれ全てのホイルシリンダ 4 4 .. について A B S 制御が実行されていない状況下で B A 制御が開始された場合に実現される増圧勾配 dB/dt 、フロントの 1 輪につき A B S 制御が実行されている状況下で B A 制御が開始された場合に実現される増圧勾配 dB/dt 、および、フロントの 2 輪につき A B S 制御が実行されている状況下で B A 制御が開始された場合に実現される増圧勾配 dB/dt を表している。

尚、図 4 に示される折れ線のうち、ほぼ傾きが“0”の領域は、ホイルシリンダ圧 $P_{w/c}$ の昇圧が開始された後、ホイルシリンダ圧 $P_{w/c}$ が急昇圧されている領域に相当する。また、図 4 に示される折れ線のうち、負の傾きを有する領域は、ホイルシリンダ圧 $P_{w/c}$ が、液圧源の液圧に近づいて収束しつつある領域を示す。

図 4 に示す如く、リアのオイルシリンダ圧 $P_{w/c}$ は、通常制御時に比して B A 制御時に大きな増圧勾配 dB/dt を示す。また、B A 制御時においては、B A 制御が開始されるに先立って、全てのオイルシリンダ 4 4 .. について A B S 制御が実行されていない場合に比して、フロントの 1 輪で A B S 制御が開始されている場合の方が大きな増圧勾配 dB/dt を示す。更に、B A 制御が開始されるに先立って、フロントの 1 輪で A B S 制御が開始されている場合に比して、フロント 2 輪で A B S 制御が実行されているとき場合に、より大きな増圧勾配 dB/dt を示す。

10 上述の如く、A B S 制御の制御対象とされているオイルシリンダ 4 4 .. は、実質的に液圧源から切り離された状態に維持される。このため、B A 制御が開始される時点でフロントの 1 輪について A B S 制御が開始されている場合は、B A 制御が開始された後に、その 1 輪のオイルシリンダにはアキュムレータ圧 P_{acc} が到達しない。

15 この場合、B A 制御が開始された後アキュムレータ 2 5 から流出するブレーキフルードは、左右後輪 R L, R R のオイルシリンダ 4 4 R L, R R とフロントの 1 輪のオイルシリンダ 4 4 F L または 4 4 F R にのみ供給される。以下、この場合を 3 輪増圧の場合と称す。

また、B A 制御が開始される時点でフロントの 2 輪について A B S 制御が開始されている場合は、B A 制御が開始された後に、フロント 2 輪のオイルシリンダにはアキュムレータ圧 P_{acc} が到達しない。この場合、B A 制御が開始された後アキュムレータ 2 5 から流出するブレーキフルードは、左右後輪 R L, R R のオイルシリンダ 4 4 R L, R R のみに供給される。尚、以下、この場合を 2 輪増圧

20 25 の場合と称す。

アキュムレータ 2 5 には、B A 制御が開始された後、4 つの車輪のオイルシリンダ 4 4 .. を迅速に昇圧するに足るブレーキフルードが貯留されている。このため、3 輪増圧の場合は、全てのオイルシリンダ 4 4 .. にブレーキフルードが流入し得る場合（以下、この場

合と4輪増圧の場合と称す)に比して、左右後輪RL, RRのオイルシリンダ44..に、より急激な圧力上昇が生ずる。同様に、2輪増圧の場合は、ブレーキフルードの供給を受ける左右後輪RL, RRのオイルシリンダ44..に、3輪増圧の場合に比して更に急激な
5 圧力上昇が生ずる。

図5は、2輪増圧または3輪増圧が実行されることにより、後輪のオイルシリンダ44RL(44RRについても同様)において実現されるオイルシリンダ圧 P_{wc} の変化を示す。図5に示すオイルシリンダ圧 P_{wc} の変化は、時刻 t_0 にブレーキ操作が開始され、
10 時刻 t_1 に2輪増圧または3輪増圧によるBA制御が開始され、更に、時刻 t_0 に、オイルシリンダ44RLについてABS制御の実行条件が成立すると判別された場合に実現される。

上述の如く、BA制御によって2輪増圧または3輪増圧が行われる場合は、4輪増圧が行われる場合に比してオイルシリンダ44RLに急激なオイルシリンダ圧 P_{wc} 上昇が生ずる。このため、この
15 ような場合は、時刻 t_0 にABS制御の実行条件の成立が判別された後に、オイルシリンダ圧 P_{wc} がABS制動油圧を大きく超える現象、すなわち、オイルシリンダ圧 P_{wc} のオーバーシュートが生ずる。

20 上述の如く、EUC10は、ABS制御の実行条件が成立した際に急激なスリップ率の増加が伴っているときには、初回の減圧時間を比較的長い時間に設定する。このため、図5に示すようなオイルシリンダ圧 P_{wc} のオーバーシュートが生ずると、ABS制御が開始された直後に、ECU20は、比較的長期間にわたって減圧モード
25 ドを実行する。

上記の如く減圧モードが長時間維持されると、オイルシリンダ44RLのオイルシリンダ圧 P_{wc} は過渡に小さな圧力に減圧されて、後輪RLが発生する制動力が不当に小さな値となることがある。このように、図1に示すシステムにおいては、BA制御が開始される

時点でフロントの1輪若しくはフロントの2輪で既にABS制御が開始されていると、BA制御に続いて後輪RL, RRについてABS制御が開始された後に、後輪RL, RRで発生される制動力が一時的に過少となる現象（以下、この現象をG抜け現象と称す）が生ずる場合がある。

本実施例の制動力制御装置は、BS制御が開始される時点で、このようなG抜け現象が生ずるのを防止する点に特徴を有している。ところで、上述したG抜け現象は、BA制御が開始される時点で後輪RL, RRについてABS制御が実行されている場合にも生じ得る。すなわち、BA制御が開始されるに先立ってリアの1輪または2輪についてABS制御が実行されている場合は、BA制御が開始されると同時に前輪FL, FRのホイールシリンダ44FL, FRにおいてホイールシリンダ圧 $P_{w/c}$ のオーバーシュートが発生する。このように前輪FL, FRのホイールシリンダ圧 $P_{w/c}$ がオーバーシュートを示せば、その後開始されるABS制御によって、前輪FL, FRのホイールシリンダ圧 $P_{w/c}$ が大きく減圧されることになる。

しかしながら、前輪FL, FRのホイールシリンダ44FL, 44FRには、後輪RL, RRのホイールシリンダ44RL, 44RRに比して大きな容量が与えられている。このため、BA制御に先立って後輪RL, RRのABS制御が実行されていても、BA制御の開始後に前輪FL, FRのホイールシリンダ圧 $P_{w/c}$ は、さほど大きくオーバーシュートすることがない。ホイールシリンダ圧 $P_{w/c}$ のオーバーシュート量がさほど大きくない場合は、その後開始されるABS制御によって前輪FL, FRのホイールシリンダ圧 $P_{w/c}$ が不当に大きく減圧されること、すなわち、大きなG抜けが生ずることがない。このため、本実施例においては、BA制御の実行に先立って前輪FL, FRについてABS制御が開始されている場合にのみ、G抜けを防止するための処理を実行している。

図6は、上記の機能を実現すべくECU20が実行する制御ルー

チンのフローチャートを示す。図 6 に示されるルーチンは、所定時間毎に起動される定時割り込みルーチンである。本ルーチンが起動されると、ステップ 100 の処理が実行される。

5 ステップ 100 では、BA 制御が実行中であるか否かが判別される。ECU 20 は、STR 28 がオン状態である場合に、BA 制御の実行中であると判別する。本ルーチンは、BA 制御の開始時に後輪 RL, RR のオイルシリンダ圧 $P_{w/c}$ にオーバーシュートが生ずるのを防止するためのルーチンである。従って、既に BA 制御が開始されている場合は、本ルーチンの処理を進める実益がない。このため、上記の判別がなされた場合は、以後何ら処理が進められることなく今回のルーチンが終了される。一方、本ステップにおいて BA 制御が非実行中である、すなわち、STR 28 がオフ状態であると判別された場合は、次にステップ 102 の処理が実行される。

10 ステップ 102 では、BA 制御の開始タイミングが到来しているか否かが判別される。その結果、未だ BA 制御の開始タイミングが到来していないと判別された場合は、以後、何ら処理が進められることなく今回のルーチンが終了される。一方、BA 制御の開始タイミングが到来していると判別された場合は、次にステップ 104 の処理が実行される。

15 ステップ 104 では、フロント 2 輪のうち少なくとも 1 輪について ABS 制御が実行されているか否かが判別される。具体的には、SA-1 46 および SA-2 48 のうち少なくとも一方がオン状態とされているか否かが判別される。上記の条件が不成立である場合は、BA 制御が開始されても、後輪 RL, RR のオイルシリンダ圧 $P_{w/c}$ に不当な急昇圧は生じないと判断することができる。この場合、次にステップ 106 において通常の BA 制御が開始された後、今回のルーチンが終了される。

20 一方、上記ステップ 104 で、フロント 2 輪のうち少なくとも 1 輪について ABS 制御が実行されていると判別された場合は、BA

制御が開始された後、後輪RL, RRのオイルシリンダ圧 $P_{w/c}$ が急昇圧されて不当にオーバーシュートする可能性がある判断できる。このため、かかる判別がなされた場合は、後輪RL, RRのオイルシリンダ圧 $P_{w/c}$ のオーバーシュートを防止すべく、次にステップ108の処理が実行される。

5 ステップ108では、BA勾配抑制制御が開始される。

BA勾配抑制制御は、通常のBA制御を実現するために実行すべき処理に加え、後輪RL, RRのオイルシリンダ44RL, RRに連通するSRRH60およびSRLH70を所定周期でオン・オフすることにより実現される。通常のBA制御を実現するための処理が実行されると、後輪RL, RRのオイルシリンダ44RL, RRとアキュムレータ25とが導通状態となる。かかる状況下でSRRH60およびSRLH70が周期的にオン・オフされると、アキュムレータ25とオイルシリンダ44RL, 44RRとが断続的に遮断状態とされ、オイルシリンダ44RL, 44RRへ流入するブレーキフルードの量が抑制される。このため、BA勾配 dB/dt 抑制制御によれば、後輪RL, RRのオイルシリンダ圧 $P_{w/c}$ が不当に急昇圧されるのを防止することができる。本ステップ108の処理が終了すると、今回のルーチンが終了される。

20 図7は、上記の処理が実行されることにより、後輪のオイルシリンダ44RL(44RRについても同様)において実現されるオイルシリンダ圧 $P_{w/c}$ の変化を示す。尚、図7中に一点鎖線で示すオイルシリンダ圧 $P_{w/c}$ の変化は、上記図5に示す特性図と同様に、BA勾配抑制制御が実行されない場合に実現されるオイルシリンダ
25 圧 $P_{w/c}$ 変化を示す。

図7中に実線で示す変化は、時刻 t_0 にブレーキ操作が開始され、少なくともフロント1輪についてABS制御が開始された後、時刻 t_{10} にBA勾配抑制制御が開始され、更に、時刻 t_{11} に、オイルシリンダ44RLについてABS制御の実行条件が成立すると判別さ

れた場合に実現される。

上述の如く、BA勾配抑制制御によれば、BA制御の実行条件が成立する時点でフロントの1輪若しくは2輪についてABS制御が開始されていても、後輪RL, RRのホイールシリンダ圧 $P_{w/c}$ を緩やかに昇圧させることができる。このため、BA勾配抑制制御が実行される場合は、後輪RL, RRのホイールシリンダ圧 $P_{w/c}$ に、ABS作動油圧を大きく超えるオーバーシュートが生ずることがない。また、ホイールシリンダ圧 $P_{w/c}$ にオーバーシュートが生じなければ、後輪RL, RRについてABS制御が開始された後に、そのホイールシリンダ圧 $P_{w/c}$ が過剰に減少されることがない。このため、本実施例の制動力制御装置によれば、フロントの1輪若しくは2輪についてABS制御が開始された後にBA制御の実行条件が成立した場合に、G抜けが生ずるのを防止することができる。従って、本実施例の制動力制御装置によれば常に良好な制御性を維持することができる。

ところで、上記の実施例においては、BA制御が開始されるに先立って実行される制動力制御をABS制御に限定しているが、本発明はこれに限定されるものではない。すなわち、本発明は、ABS制御に代えて、ホイールシリンダの液圧流入経路を遮断した状態でホイールシリンダ圧 $P_{w/c}$ を制御する他の制御液圧減圧制御が用いられる場合にも適用が可能である。

また、上記の実施例においては、“前輪FL, FRについて”ABS制御が実行されている場合に“後輪RL, RRについて”BA勾配抑制制御を実行することとしているが、本発明はこれに限定されるものではない。すなわち、何れかのホイールシリンダについてABS制御等の制動液圧減圧制御が実行されている場合に、他のホイールシリンダについてBA勾配抑制制御を実行するものであればよい。

尚、上記の実施例においては、調圧用液圧通路56, 62が「液圧流入経路」に、ABS制御が「制動液圧減圧制御」に、それぞれ

相当していると共に、ECU 20 が上記ステップ 104 の処理を実行することにより導通検出手段が、また、ECU 20 が上記ステップ 108 の処理を実行することにより液圧流入抑制手段が、それぞれ実現されている。

- 5 次に、図 8 および図 9 を参照して、本発明の第 2 実施例について説明する。本実施例の制動力制御装置は、上記図 1 に示すシステムにおいて、ECU 20 に、上記図 6 に示すルーチンに代えて、図 8 に示すルーチンを実行させることにより実現される。

上述した第 1 実施例の制動力制御装置は、BA 制御の実行条件が
10 成立するに先立って少なくともフロントの 1 輪について ABS 制御が実行されている場合に、BA 制御の開始に伴うホイルシリンダ圧 $P_{w/c}$ の上昇率を下げることでホイルシリンダ圧 $P_{w/c}$ のオーバーシュートを抑制している。しかし、BA 制御は、緊急ブレーキを要求する操作が行われた際に、速やかにホイルシリンダ圧 $P_{w/c}$ を立
15 ち上げることを目的として実行される制御である。この点、上述した第 1 実施例が用いる手法は、BA 制御本来の目的と背反している。

本実施例は、BA 制御の実行条件が成立するに先立って少なくともフロントの 1 輪について ABS 制御が開始されている場合に、BA 制御の開始に伴うホイルシリンダ圧 $P_{w/c}$ の上昇率を低下させる
20 ことなく、ホイルシリンダ圧 $P_{w/c}$ のオーバーシュートを防止する点に特徴を有している。

図 8 は、上記の機能を実現すべく ECU 20 が実行するルーチンの一例のフローチャートを示す。本ルーチンは、後輪 RL, RR について ABS 制御を開始するタイミングを判断するために実行される
25 ルーチンである。本ルーチンは、所定時間毎に起動される定時割り込みルーチンである。本ルーチンが起動されると、ステップ 110 の処理が実行される。

ステップ 110 では、BA 制御が実行されているか否かが判別される。具体的には、STR 28 がオン状態であるか否かが判別され

る。その結果、STR 28 がオフ状態であると判別された場合は、BA 制御が実行されていないと判断される。この場合、次にステップ 120 の処理が実行される。

5 ステップ 120 では、後輪 RL, RR のスリップ量が所定値 ΔV_1 に比して大きいかが判別される。 ΔV_1 は、車輪がロック状態に以降する直前のスリップ量である。上記の判別の結果、後輪 RL, RR のスリップ量が ΔV_1 を超えていると判別された場合は、後輪 RL, RR について ABS 制御を開始すべきであると判断される。この場合、次にステップ 122 の処理が実行される。一方、本
10 ステップ 120 で後輪 RL, RR のスリップ量が ΔV_1 以下であると判別された場合は、ABS 制御を開始する必要がないと判断され、そのまま今回の処理が終了される。

 ステップ 122 では、通常 ABS 制御を開始するための処理が実行される。本ステップ 122 の処理が実行されると、以後、上述した ABS 制御、すなわち、②減圧モード→③保持モード→①増圧
15 モード→④緩増圧モードを繰り返す処理が開始される。本ステップ 122 の処理が終了すると、今回のルーチンが終了される。

 上記ステップ 110 において、BA 制御が実行されている、すなわち、STR 28 がオン状態であると判別された場合は、次にス
20 テップ 112 の処理が実行される。ステップ 112 では、フロント 2 輪のうち少なくとも 1 輪について ABS 制御が実行されているか否か、すなわち、SA-1 46 および SA-2 48 の少なくとも一方がオン状態とされているか否かが判別される。その結果、上記の条件が不成立であると判別される場合は、BA 制御の実行に伴って後輪
25 RL, RR のオイルシリンダ圧 $P_{w/c}$ に通常時と異なる急昇圧は生じていないと判断することができる。この場合、通常の条件で ABS 制御の実行判定を行うべく、次にステップ 120 の処理が実行される。

 一方、上記ステップ 112 で、フロント 2 輪のうち少なくとも 1

輪についてABS制御が実行されていると判別された場合は、BA制御の実行に伴って、後輪RL, RRのオイルシリンダ圧 $P_{w/c}$ に、通常時に比して急激な昇圧が生じていると判断することができる。この場合、次にステップ114の処理が実行される。

- 5 ステップ114では、後輪RL, RRのスリップ量が所定値 ΔV_2 に比して大きいかが判別される。 ΔV_2 は、上記ステップ120で用いられるしきい値 ΔV_1 に比して小さな値、すなわち、車輪がロック状態に移行するスリップ量に比して小さな値である。上記の判別の結果、後輪RL, RRのスリップ量が ΔV_2 以下であると判別された場合は、未だ後輪RL, RRのオイルシリンダ圧 $P_{w/c}$ がABS作動油圧に比して十分に小さいと判断することができる。この場合、以後、何ら処理が進められることなく今回のルーチンが終了される。

- 15 一方、上記ステップ114で、後輪RL, RRのスリップ量が ΔV_2 を超えていると判別された場合は、後輪RL, RRのオイルシリンダ圧 $P_{w/c}$ がABS作動油圧の近傍にまで昇圧されていると判断することができる。この場合、次にステップ116の処理が実行される。

- 20 ステップ116では、後輪RL, RRについてのABS制御が既に開始されているかが判別される。後輪RL, RRについてのABS制御が未だ開始されていないと判別される場合は、後輪RL, RRのオイルシリンダ圧 $P_{w/c}$ の昇圧特性がBA制御によって支配されている、すなわち、そのオイルシリンダ圧 $P_{w/c}$ が急激に昇圧されていると判断することができる。この場合、次にステップ118の処理が実行される。

25 一方、上記ステップ116で、後輪RL, RRについてのABS制御が既に開始されていると判別される場合は、後輪RL, RRのオイルシリンダ圧 $P_{w/c}$ の昇圧特性がABS制御によって支配されている、すなわち、そのオイルシリンダ圧 $P_{w/c}$ はもはや急昇圧し

ていないと判断することができる。この場合、以後、通常のABS制御を続行すべく、次にステップ120の処理が実行される。

- 5 ステップ118では、初回特定ABS制御を開始するための処理が実行される。初回特定ABS制御は、ABS制御の開始直後に実行される②減圧モードの実行時間を、通常ABS制御中で実行される②減圧モードの実行時間に比して長期化した制御である。初回特定ABS制御によれば、通常ABS制御に比して、後輪RL, RRのホイールシリンダ圧 $P_{w/c}$ を大きく減圧することができる。本ステップ118の処理が終了すると、今回のルーチンが終了される。
- 10 上記の処理によれば、BA制御の開始に先立って少なくともフロント1輪についてABS制御が開始されている場合においても、BA制御が開始された後、後輪RL, RRのスリップ量が ΔV_2 を超えるまでは、後輪RL, RRのホイールシリンダ圧 $P_{w/c}$ を急昇圧させることができる。また、急昇圧されたホイールシリンダ圧 $P_{w/c}$ が
- 15 ABS作動油圧の近傍にまで昇圧されると、その時点で、初回特定ABS制御によって後輪RL, RRのホイールシリンダ圧 $P_{w/c}$ の減圧を開始することができる。更に、初回特定ABS制御によれば、②減圧モードが長時間維持されるため、急昇圧されていたホイールシリンダ圧 $P_{w/c}$ を適切に減圧することができる。このため、本実施
- 20 例の制動力制御装置によれば、BA制御が開始された後に後輪RL, RRのホイールシリンダ圧 $P_{w/c}$ がオーバーシュートするのを確実に防止することができる。

- 25 図9は、上記の処理が実行されることにより、後輪のホイールシリンダ44RL(44RRについても同様)において実現されるホイールシリンダ圧 $P_{w/c}$ の変化を示す。尚、図9中に一点鎖線で示すホイールシリンダ圧 $P_{w/c}$ の変化は、上記図5に示す特性図と同様に、通常のBA制御が開始された後に通常のABS制御が開始された場合に実現されるホイールシリンダ圧 $P_{w/c}$ 変化を示す。

図9中に実線で示す変化は、時刻 t_{12} にブレーキ操作が開始され、

少なくともフロント1輪についてABS制御が開始された後、時刻 t_{13} にBA制御が開始され、更に、時刻 t_{14} に、ホイールシリンダ44RLについて初回特定ABS制御の実行条件が成立する、すなわち、後輪RLのスリップ量が ΔV_2 を超えていると判別された場合に実現される。

少なくともフロント1輪についてABS制御が開始された状態でBA制御が開始されると、その後、後輪RLのホイールシリンダ圧 $P_{w/c}$ は急激に上昇する。一方、初回特定ABS制御によれば、通常ABS制御の場合に比して早期に、かつ、大きくホイールシリンダ44RLのホイールシリンダ圧 $P_{w/c}$ を減圧させることができる。このため、本実施例の制動力制御装置においては、後輪RL、RRのホイールシリンダ圧 $P_{w/c}$ に、ABS作動油圧を大きく超えるオーバーシュートが生ずることがない。このため、本実施例の制動力制御装置によれば、フロントの1輪若しくは2輪についてABS制御が開始された後にBA制御の実行条件が成立した場合に、優れた制御性を維持することができる。

ところで、上記の実施例においては、BA制御が開始されるに先立って実行される制動力制御をABS制御に限定しているが、本発明はこれに限定されるものではない。すなわち、本発明は、ABS制御に代えて、先ずホイールシリンダの液圧流入経路を遮断した状態でホイールシリンダ圧 $P_{w/c}$ の減圧を図り、次いで所望の液圧制御を実行する他の制動液圧制御が用いられる場合にも適用が可能である。

また、上記の実施例においては、“前輪FL、FRについて”ABS制御が実行されている場合に“後輪RL、RRについて”初回特定ABS制御を実行することとしているが、本発明はこれに限定されるものではない。すなわち、何れかのホイールシリンダについてABS制御等の制動液圧制御が実行されている場合に、他のホイールシリンダについて初回特定ABS制御を実行する場合にも適用が可能である。

尚、上記の実施例においては、車輪のスリップ量が「車輪のス
リップ状態に関する特性値」に、調圧用液圧通路 56, 62 が「液
圧流入経路」に、ABS 制御が「制動液圧制御」に、ABS 制御中
5 で始めて減圧モードを実現する制御が「減圧制御」に、それぞれ相
当していると共に、ECU 20 が上記ステップ 112 の処理を実行
することにより「導通検出手段」が、ECU 20 が上記ステップ 1
18 の処理を実行することにより「しきい値変更手段」および「減
圧傾向変更手段」が、それぞれ実現されている。

次に、図 10 および図 11 を参照して、本発明の第 3 実施例につ
10 いて説明する。本実施例の制動力制御装置は、上記図 1 に示すシ
ステム構成において、ECU 20 に、上記図 6 または図 8 に示すルー
チンと共に、または、上記図 6 または図 8 に示すルーチンに代えて、
図 11 に示す制御ルーチンを実行させることにより実現される。

ABS 制御の実行に伴うホイールシリンダ圧 $P_{w/c}$ の増圧勾配は、
15 ホイールシリンダ 44.. に液圧を供給する液圧源の液圧（すなわち、
レギュレータ圧 P_{RE} またはアキュムレータ圧 P_{Acc} ）とホイールシ
リンダ圧 $P_{w/c}$ との差圧、液圧通路やソレノイドバルブの有効径、お
よび、保持ソレノイド $S_{..H}$ の開弁時間等により決定される。BA
制御が実行されないシステムにおいては、液圧源や液圧通路の特性
20 に変化が生ずることはない。かかるシステムでは、ABS 制御の内
容は、それらの特性が固定されていることを前提としてチューニン
グされる。

しかし、BA 制御が実行されるシステムにおいては、BA 制御の
実行に伴って液圧源や液圧通路が変更される。このため、かかるシ
25 ステムにおいては、ABS 制御が単独で実行されている場合と、A
BS 制御が BA 制御と共に実行されている場合とで、ホイールシリン
ダ圧 $P_{w/c}$ に異なる増圧勾配が与えられる。ABS 制御に伴うホイ
ールシリンダ圧 $P_{w/c}$ の増圧勾配が変化すると、ABS 制御の制御特
性に変化が生じ、常に同様の制動特性を得ることができなくなる。

ところで、B A制御が実行されるシステムにおいても、B A制御が実行されているか否かに対応してA B S制御の内容を切り換えれば、B A制御が実行中か否かに関わらず、A B S制御の実行中に同様の増圧勾配を得ることが可能である。本実施例の制動力制御装置は、B A制御の実行状態に応じて、A B S制御の設定条件を変更することで、上記の機能を実現する点に特徴を有している。

図10は、図1に示すシステムにおいてB A制御の実行・停止に伴う液圧源および増圧特性の変化を示す。図10に示す如く、本実施例のシステムにおいてB A制御が実行されていない間は、レギュレータ27がA B S制御時の液圧源となる。この際、本実施例のシステムでは、レギュレータ27の液圧吐出能力、レギュレータ27と第3液圧通路42とを結ぶ通路の特性、および、第3液圧通路42下流の特性に応じた増圧特性（以下、この増圧特性を特性①と称す）が実現される。

また、図10に示す如く、本実施例のシステムにおいてB A制御が実行されている間は、アキュムレータ25がA B S制御時の液圧源となる。この際、本実施例のシステムでは、ポンプ21およびアキュムレータ25の液圧吐出能力、アキュムレータ25から第3液圧通路42に至る通路の特性、および、第3液圧通路42下流の特性に応じた増圧特性（以下、この増圧特性を特性②と称す）が実現される。

図11は、特性①と特性②とを同一にすべくE C U 20が実行する制御ルーチンの一例のフローチャートを示す。本ルーチンは、所定時間毎に起動される定時割り込みルーチンである。本ルーチンが起動されると、先ずステップ130の処理が実行される。

ステップ130では、B A制御が実行中であるか否かが判別される。本ステップ130では、S T R 28の状態に基づいて上記の判別がなされる。具体的には、S T R 28がオフ状態である場合はB A制御が実行されていないと、また、S T R 28がオン状態である

場合はB A制御が実行されていると判断される。B A制御が実行されていないと判断された場合は、次にステップ1 3 2の処理が実行される。一方、B A制御が実行されていると判断された場合は、次にステップ1 3 4の処理が実行される。

- 5 ステップ1 3 2では、A B S制御の駆動条件を条件①とする処理が実行される。条件①は、レギュレータ2 7が、制御液圧通路3 0およびS T R 2 8を介して第3液圧通路4 2に連通されている場合に、特性①を所望の増圧勾配とするための条件である。本ステップ1 3 2の処理が終了すると、今回のルーチンが終了される。上記ス
10 テップ1 3 2の処理が実行されると、以後、A B S制御は、条件①に従って実行される。

- ステップ1 3 4では、A B S制御の駆動条件を条件②とする処理が実行される。条件②は、アキュムレータ2 5が、高圧通路2 6およびS T R 2 8を介して第3液圧通路4 2に連通されている場合に、
15 特性②を所望の増圧勾配とするための条件である。本ステップ1 3 4の処理が終了すると、今回のルーチンが終了される。上記ステップ1 3 4の処理が実行されると、以後、A B S制御は、条件②に従って実行される。

- 上記の処理によれば、B A制御が実行中であると否とに関わらず、
20 A B S制御の実行に伴って、常に同様に所望の増圧特性でホイールシリンダ圧 $P_{w/c}$ を昇圧させることが可能となる。このため、本実施例の制動力制御装置によれば、B A制御の実行に伴ってA B S制御の制御性が悪化するという不都合を回避することができる。

- 本実施例では、条件①および②により、保持ソレノイドS..Hの
25 駆動パターンを決めることとしている。より具体的には、保持ソレノイドS..Hの駆動パターンを決めるマップを2種類準備し、条件①および②で何れのマップを用いるかを決定することとしている。尚、保持ソレノイドS..Hの駆動パターンを切り換える手法はこれに限定されるものではなく、基準のマップに補正を施すか否かによ

り、その駆動パターンを切り換えることとしてもよい。

更に、上記の実施例においては、条件①および②で決定すべき内容が保持ソレノイド S..H の駆動パターンに限定されているが、条件①および②で決定すべき内容はこれに限定されるものではなく、
5 例えば、液圧源の特性を変更することで、B A 制御の実行中と非実行中とで同一の増圧勾配を実現することとしてもよい。

次に、図 1 2 乃至図 2 2 を参照して、本発明の第 4 実施例による制動力制御装置について説明する。

図 1 2 は、本発明の第 4 実施例によるポンプアップ式制動力制御
10 装置（以下、単に制動力制御装置と称す）のシステム構成図を示す。本実施例の制動力制御装置は、フロントエンジン・リアドライブ式車両（F R 車両）用の制動力制御装置として好適な装置である。本実施例の制動力制御装置は、電子制御ユニット 2 1 0 （以下、E C U 2 1 0 と称す）により制御されている。

15 制動力制御装置は、ブレーキペダル 2 1 2 を備えている。ブレーキペダル 2 1 2 の近傍には、ブレーキスイッチ 2 1 4 が配設されている。ブレーキスイッチ 2 1 4 は、ブレーキペダル 2 1 2 が踏み込まれることによりオン信号を出力する。ブレーキスイッチ 2 1 4 の出力信号は E C U 2 1 0 に供給されている。E C U 2 1 0 は、
20 レーキスイッチ 2 1 4 の出力信号に基づいてブレーキペダル 2 1 2 が踏み込まれているか否かを判別する。

ブレーキペダル 2 1 2 は、バキュームブースタ 2 1 6 に連結されている。バキュームブースタ 2 1 6 は、マスタシリンダ 2 1 8 に固定されている。バキュームブースタ 2 1 6 は、ブレーキペダル 2 1
25 2 が踏み込まれた場合に、ブレーキ踏力 F に対して所定の倍力比を有するアシスト力 F a を発生する。マスタシリンダ 2 1 8 は、センタバルブ・コンベンショナルタイプのマスタシリンダであり、その内部に第 1 油圧室 2 2 0 および第 2 油圧室 2 2 2 を備えている。第 1 油圧室 2 2 0 および第 2 油圧室 2 2 2 には、ブレーキ踏力 F と

アシスト力 F_a との合力に応じたマスタシリンダ圧 $P_{m/c}$ が発生する。

マスタシリンダ 218 の上部にはリザーバタンク 224 が配設されている。リザーバタンク 224 には、フロントリザーバ通路 226、および、リアリザーバ通路 228 が連通している。フロントリザーバ通路 226 には、フロントリザーバカットソレノイド 230（以下、SRCF 230 と称す）が連通している。同様に、リアリザーバ通路 228 には、リアリザーバカットソレノイド 232（以下、SRCR 232 と称す）が連通している。

- 10 SRCF 230 には、更に、フロントポンプ通路 234 が連通している。同様に、SRCR 232 には、リアポンプ通路 236 が連通している。SRCF 230 は、オフ状態とされることでフロントリザーバ通路 226 とフロントポンプ通路 234 とを遮断し、かつ、オン状態とされることでそれらを導通させる 2 位置の電磁弁である。
- 15 また、SRCR 232 は、オフ状態とされることでリアリザーバ通路 228 とリアポンプ通路 236 とを遮断し、かつ、オン状態とされることでそれらを導通させる 2 位置の電磁弁である。

マスタシリンダ 218 の第 1 油圧室 220、および、第 2 油圧室 222 には、それぞれ第 1 液圧通路 238、および、第 2 液圧通路 240 が連通している。第 1 液圧通路 238 には、右前マスタカットソレノイド 242（以下、SMFR 242 と称す）、および、左前マスタカットソレノイド 244（以下、SMFL 244 と称す）が連通している。一方、第 2 液圧通路 240 には、リアマスタカットソレノイド 246（以下、SMR 246 と称す）が連通している。

- 25 SMFR 242 には、右前輪 FR に対応して設けられた液圧通路 248 が連通している。同様に、SMFL 244 には、左前輪 FL に対応して設けられた液圧通路 250 が連通している。更に、SMR 246 には、左右後輪 RL, RR に対応して設けられた液圧通路 252 が連通している。

SMFR 2 4 2、SMFL 2 4 4 および SMR 2 4 6 の内部には、それぞれ定圧開放弁 2 5 4、2 5 6、2 5 8 が設けられている。SMFR 2 4 2 は、オフ状態とされた場合に第 1 液圧通路 2 3 8 と液圧通路 2 4 8 とを導通状態とし、かつ、オン状態とされた場合に定圧開放弁 2 5 4 を介して第 1 液圧通路 2 3 8 と液圧通路 2 4 8 とを連通させる 2 位置の電磁弁である。また、SMFL 2 4 2 は、オフ状態とされた場合に第 1 液圧通路 2 3 8 と液圧通路 2 5 0 とを導通状態とし、かつ、オン状態とされた場合に定圧開放弁 2 5 6 を介して第 1 液圧通路 2 3 8 と液圧通路 2 5 0 とを連通させる 2 位置の電磁弁である。同様に、SMR 2 4 6 は、オフ状態とされた場合に第 2 液圧通路 2 4 0 と液圧通路 2 5 2 とを導通状態とし、かつ、オン状態とされた場合に定圧開放弁 2 5 8 を介して第 2 液圧通路 2 4 0 と液圧通路 2 5 2 とを連通させる 2 位置の電磁弁である。

第 1 液圧通路 2 3 8 と液圧通路 2 4 8 との間には、また、第 1 液圧通路 2 3 8 側から液圧通路 2 4 8 側へ向かうフルードの流れのみを許容する逆止弁 2 6 0 が配設されている。同様に、第 1 液圧通路 2 3 8 と液圧通路 2 5 0 との間、および、第 2 液圧通路 2 4 0 と液圧通路 2 5 2 との間には、それぞれ第 1 液圧通路 2 3 8 側から液圧通路 2 5 0 側へ向かう流体の流れのみを許容する逆止弁 2 6 2、および、第 2 液圧通路 2 4 0 側から液圧通路 2 5 2 側へ向かう流体の流れのみを許容する逆止弁 2 6 4 が配設されている。

右前輪 FR に対応する液圧通路 2 4 8 には、右前輪保持ソレノイド 2 6 6 (以下、SFRH 2 6 6 と称す) が連通している。同様に、左前輪 FL に対応する液圧通路 2 5 0 には左前輪保持ソレノイド 2 6 8 (以下、SFLH 2 6 8 と称す) が、左右後輪 RL、RR に対応する液圧通路 2 5 2 には右後輪保持ソレノイド 2 7 0 (以下、SRRH 2 7 0 と称す) および左後輪保持ソレノイド 2 7 2 (以下、SRLH 2 7 2 と称す) が、それぞれ連通している。以下、これらのソレノイドを総称する場合は「保持ソレノイド S ** H」と称す。

SFRH266には、右前輪減圧ソレノイド274（以下、SFRH274と称す）が連通している。同様に、SFLH268、SRRH270およびSRLH272には、それぞれ左前輪減圧ソレノイド276（以下、SFLR276と称す）、右後輪減圧ソレノイド278（以下、SRRR278と称す）および左後輪減圧ソレノイド280（以下、SRLR280と称す）が、それぞれ連通している。以下、これらのソレノイドを総称する場合には「減圧ソレノイドS**R」と称す。

SFRH266には、また、右前輪FRのオイルシリンダ282が連通している。同様に、SFLH268には左前輪FLのオイルシリンダ284が、SRRH270には右後輪RRのオイルシリンダ286が、また、SRLH272には左後輪RLのオイルシリンダ288がそれぞれ連通している。

更に、液圧通路248とオイルシリンダ282との間には、SFRH266をバイパスしてオイルシリンダ282側から液圧通路248へ向かうフルードの流れを許容する逆止弁290が配設されている。同様に、液圧通路250とオイルシリンダ284との間、液圧通路252とオイルシリンダ286との間、および、液圧通路252とオイルシリンダ288との間には、それぞれSFLH268、SRRH270およびSRLH272をバイパスするフルードの流れを許容する逆止弁292，294，296が配設されている。

SFRH266は、オフ状態とされることにより液圧通路248とオイルシリンダ282とを導通状態とし、かつ、オン状態とされることにより液圧通路248とオイルシリンダ282とを遮断状態とする2位置の電磁弁である。同様に、SFLH268、SRRH270およびSRLH272は、それぞれオン状態とされることにより液圧通路250とオイルシリンダ284とを結ぶ経路、液圧通路252とオイルシリンダ286とを結ぶ経路、および、液圧通路252とオイルシリンダ288とを結ぶ経路を遮断する2位置の電磁弁で

ある。

左右前輪の減圧ソレノイドSFRR274およびSFLR276
には、フロント減圧通路298が連通している。また、左右後輪の
減圧ソレノイドSRRR278およびSRLR280にはリア減圧
5 通路300が連通している。フロント減圧通路298およびリア減
圧通路300には、それぞれフロントリザーバ302およびリアリ
ザーバ304が連通している。

また、フロント減圧通路298およびリア減圧通路300は、そ
れぞれ逆止弁306、308を介してフロントポンプ310の吸入
10 側、および、リアポンプ312の吸入側に連通している。フロント
ポンプ310の吐出側、および、リアポンプ312の吐出側は、吐
出圧の脈動を吸収するためのダンパ314、316に連通している。
ダンパ314は、右前輪FRに対応して設けられた右前ポンプ通路
318および左前輪FLに対応して設けられた左前ポンプ通路32
15 0に連通している。一方、ダンパ316は、液圧通路252に連通
している。

右前ポンプ通路318は、右前ポンプソレノイド322（以下、
SPFL322と称す）を介して液圧通路248に連通している。
また、左前ポンプ通路320は、左前ポンプソレノイド324（以
20 下、SPFR324と称す）を介して液圧通路250に連通してい
る。SPFL322は、オフ状態とされることにより右前ポンプ通
路318と液圧通路248とを導通状態とし、かつ、オン状態とさ
れることによりそれらを遮断状態とする2位置の電磁弁である。同
様に、SPFR324は、オフ状態とされることにより左前ポンプ
25 通路320と液圧通路250とを導通状態とし、かつ、オン状態と
されることによりそれらを遮断状態とする2位置の電磁弁である。

液圧通路248と右前ポンプ通路318との間には、液圧通路2
48側から右前ポンプ通路318側へ向かう流体の流れのみを許容
する定圧開放弁326が配設されている。同様に、液圧通路250

と左前ポンプ通路 3 2 0 との間には、液圧通路 2 5 0 側から左前ポンプ通路 3 2 0 側へ向かう流体の流れのみを許容する定圧開放弁 3 2 8 が配設されている。

5 各車輪の近傍には、車輪速センサ 3 3 0, 3 3 2, 3 3 4, 3 3 6 が配設されている。E C U 2 1 0 は、車輪速センサ 3 3 0 ~ 3 3 6 の出力信号に基づいて各車輪の回転速度 V_w を検出する。また、マスタシリンダ 2 1 8 に連通する第 2 液圧通路 2 4 0 には、液圧センサ 3 3 8 が配設されている。E C U 2 1 0 は、液圧センサ 3 3 8 の出力信号に基づいてマスタシリンダ圧 $P_{m/c}$ を検出する。

10 次に、本実施例の制動力制御装置の動作を説明する。本実施例の制動力制御装置は、油圧回路内に配設された各種の電磁弁の状態を切り換えることにより、①通常ブレーキ機能、②A B S 機能、および、③B A 機能を実現する。

①通常ブレーキ機能は、図 1 2 に示す如く、制動力制御装置が備える全ての電磁弁をオフ状態とすることにより実現される。以下、図 1 2 に示す状態を通常ブレーキ状態と称す。また、制動力制御装置において通常ブレーキ機能を実現するための制御を通常ブレーキ制御と称す。

図 1 2 に示す通常ブレーキ状態において、左右前輪 F L, F R の
20 ホイルシリンダ 2 8 2, 2 8 4 は、共に第 1 液圧通路 2 3 8 を介してマスタシリンダ 2 1 8 の第 1 油圧室 2 2 0 に連通している。また、左右後輪 R L, R R のホイルシリンダ 2 8 6, 2 8 8 は、第 2 液圧通路 2 4 0 を介してマスタシリンダ 2 1 8 の第 2 油圧室 2 2 2 に連通している。この場合、ホイルシリンダ 2 8 2 ~ 2 8 8 のホイルシ
25 リンダ圧 $P_{w/c}$ は、常にマスタシリンダ圧 $P_{m/c}$ と等圧に制御される。従って、図 1 2 示す状態によれば、通常ブレーキ機能が実現される。

②A B S 機能は、図 1 2 に示す状態において、フロントポンプ 3 1 0 およびリアポンプ 3 1 2 をオン状態とし、かつ、保持ソレノイ

ドS**Hおよび減圧ソレノイドS**RをABSの要求に応じて適当に駆動することにより実現される。以下、制動力制御装置においてABS機能を実現するための制御をABS制御と称す。

5 ECU 210は、車両が制動状態にあり、かつ、何れかの車輪について過剰なスリップ率が検出された場合にABS制御を開始する。ABS制御は、ブレーキペダル212が踏み込まれている状況下、すなわち、マスタシリンダ218が高圧のマスタシリンダ圧 $P_{m/c}$ を発生している状況下で開始される。

10 ABS制御の実行中は、マスタシリンダ圧 $P_{m/c}$ が、第1液圧通路238および第2液圧通路240を介して、それぞれ左右前輪に対応して設けられた液圧通路248, 250、および、左右後輪に対応して設けられた液圧通路252に導かれる。従って、かかる状況下で保持ソレノイドS**Hを開弁状態とし、かつ、減圧ソレノイドS**Rを閉弁状態とすると、各車輪のホイールシリンダ圧 $P_{w/c}$ を増圧することができる。以下、この状態を(i)増圧モードと称す。

また、ABS制御の実行中に、保持ソレノイドS**Hおよび減圧ソレノイドS**Rの双方を閉弁状態とすると、各車輪のホイールシリンダ圧 $P_{w/c}$ を保持することができる。以下、この状態を(ii)保持モードと称す。更に、ABS制御の実行中に、保持ソレノイドS**Hを閉弁状態とし、かつ、減圧ソレノイドS**Rを開弁状態とすると、各車輪のホイールシリンダ圧 $P_{w/c}$ を減圧することができる。以下、この状態を(iii)減圧モードと称す。

25 ECU 210は、ABS制御中に、各車輪毎に適宜上記の(i)増圧モード、(ii)保持モード、および、(iii)減圧モードが実現されるように、各車輪のスリップ状態に応じて保持ソレノイドS**Hおよび減圧ソレノイドS**Rを制御する。保持ソレノイドS**Hおよび減圧ソレノイドS**Rが上記の如く制御されると、全ての車輪のホイールシリンダ圧 $P_{w/c}$ が対応する車輪に過大なスリップ

率を発生させることのない適当な圧力に制御される。このように、上記の制御によれば、制動力制御装置においてABS機能を実現することができる。

ABS制御の実行中に、各車輪で減圧モードが行われる際には
5 ホイルシリンダ282～288内のブレーキフルードが、フロント減
圧通路298およびリア減圧通路300を通してフロントリザーバ
302およびリアリザーバ304に流入する。フロントリザーバ3
02およびリアリザーバ304に流入したブレーキフルードは、フ
ロントポンプ310およびリアポンプ312に汲み上げられて液圧
10 通路248, 250, 252へ供給される。

液圧通路248, 250, 252に供給されたブレーキフルード
の一部は、各車輪で増圧モードが行われる際にホイルシリンダ28
2～288に流入する。また、そのブレーキフルードの残部は、ブ
レーキフルードの流出分を補うべくマスタシリンダ218に流入す
15 る。このため、本実施例によれば、ABS制御の実行中にブレーキ
ペダル212に過大なストロークが生ずることはない。

図13乃至図15は、③BA機能を実現するための制動力制御装
置の状態を示す。ECU210は、運転者によって制動力の速やか
な立ち上がりを要求するブレーキ操作すなわち緊急ブレーキ操作が
20 実行された後に、図13乃至図15に示す状態を適宜実現すること
でBA機能を実現する。以下、制動力制御装置において、BA機能
を実現させるための制御をBA制御と称す。

図13は、BA制御の実行中に実現されるアシスト圧増圧状態を
示す。アシスト圧増圧状態は、BA制御の実行中に各車輪のホイル
25 シリンダ圧 $P_{w/c}$ を増圧させる必要がある場合に実現される。本実
施例のシステムにおいて、BA制御中におけるアシスト圧増圧状態
は、図13に示す如く、リザーバカットソレノイドSRCF230,
SRCR232、および、マスタカットソレノイドSMFR242,
SMFL244, SMR246をオン状態とし、かつ、フロントボ

ンプ 3 1 0 およびリアポンプ 3 1 2 をオン状態とすることで実現される。

図 1 3 に示すアシスト圧増圧状態が実現されると、リザーバタンク 2 2 4 に貯留されているブレーキフルードがフロントポンプ 3 1 0 およびリアポンプ 3 1 2 に汲み上げられて液圧通路 2 4 8, 2 5 0, 2 5 2 に供給される。アシスト圧増圧状態では、液圧通路 2 4 8, 2 5 0, 2 5 2 の内圧が、定圧開放弁 2 5 4, 2 5 6, 2 5 8 の開弁圧を超えてマスタシリンダ圧 $P_{m/c}$ に比して高圧となるまでは、液圧通路 2 4 8, 2 5 0, 2 5 2 からマスタシリンダ 2 1 8 へ向かうブレーキフルードの流れが SMFR 2 4 2, SMFL 2 4 4, SMR 2 4 6 によって阻止される。

このため、図 1 3 に示すアシスト圧増圧状態が実現されると、その後、液圧通路 2 4 8, 2 5 0, 2 5 2 には、マスタシリンダ圧 $P_{m/c}$ に比して高圧の液圧が発生する。アシスト圧増圧状態では、ホイールシリンダ 2 8 2 ~ 2 8 8 と、それらに対応する液圧通路 2 4 8, 2 5 0, 2 5 2 とが導通状態に維持されている。従って、アシスト圧増圧状態が実現されると、その後、全ての車輪のホイールシリンダ圧 $P_{w/c}$ は、フロントポンプ 3 1 0 またはリアポンプ 3 1 2 を液圧源として、速やかにマスタシリンダ圧 $P_{m/c}$ を超える圧力に昇圧される。

ところで、図 1 3 に示すアシスト圧増圧状態において、液圧通路 2 4 8, 2 5 0, 2 5 2 は、それぞれ逆止弁 2 6 0, 2 6 2, 2 6 4 を介してマスタシリンダ 2 1 8 に連通している。このため、マスタシリンダ圧 $P_{m/c}$ が各車輪のホイールシリンダ圧 $P_{w/c}$ に比して大きい場合は、アシスト圧増圧状態においても、マスタシリンダ 2 1 8 を液圧源としてホイールシリンダ圧 $P_{w/c}$ を昇圧することができる。

図 1 4 は、BA 制御の実行中に実現されるアシスト圧保持状態を示す。アシスト圧保持状態は、BA 制御の実行中に各車輪のホイールシリンダ圧 $P_{w/c}$ を保持する必要がある場合に実現される。アシス

ト圧保持状態は、図 1 4 に示す如く、SRCF 2 3 0, SRCR 2 3 2 をオフ状態とし、マスタカットソレノイド SMFR 2 4 2, SMFL 2 4 4, SMR 2 4 6 をオン状態とし、かつ、フロントポンプ 3 1 0 およびリアポンプ 3 1 2 をオン状態とすることで実現される。

図 1 4 に示すアシスト圧保持状態では、フロントポンプ 3 1 0 とリザーバタンク 2 2 4、および、リアポンプ 3 1 2 とリザーバタンク 2 2 4 が、それぞれ SRCF 2 3 0 および SRCR 2 3 2 によって遮断状態とされる。このため、アシスト圧保持状態では、フロントポンプ 3 1 0 およびリアポンプ 3 1 2 から液圧通路 2 4 8, 2 5 0, 2 5 2 にフルードが吐出されることはない。また、図 1 4 に示すアシスト圧保持状態では、液圧通路 2 4 8, 2 5 0, 2 5 2 が、SMFR 2 4 2, SMFL 2 4 4, SMR 2 4 6 によってマスタシリンダ 2 1 8 から実質的に切り離されている。このため、図 1 4 に示すアシスト圧保持状態によれば、全ての車輪のホイールシリンダ圧 $P_{w/c}$ を一定値に保持することができる。

図 1 5 は、BA 制御の実行中に実現されるアシスト圧減圧状態を示す。アシスト圧減圧状態は、BA 制御の実行中に各車輪のホイールシリンダ圧 $P_{w/c}$ を減圧する必要がある場合に実現される。アシスト圧減圧状態は、図 1 5 に示す如く、フロントポンプ 3 1 0 およびリアポンプ 3 1 2 をオン状態とすることで実現される。

図 1 5 に示すアシスト圧減圧状態では、フロントポンプ 3 1 0 およびリアポンプ 3 1 2 がリザーバタンク 2 2 4 から切り離される。このため、フロントポンプ 3 1 0 およびリアポンプ 3 1 2 から液圧通路 2 4 8, 2 5 0, 2 5 2 にフルードが吐出されることはない。また、アシスト圧減圧状態では、各車輪のホイールシリンダ 2 8 2 ~ 2 8 8 とマスタシリンダ 2 1 8 とが導通状態となる。このため、アシスト圧減圧状態を実現すると、全ての車輪のホイールシリンダ圧 $P_{w/c}$ を、マスタシリンダ圧 $P_{m/c}$ を下限値として減圧することがで

きる。

本実施例の制動力制御装置において、BA制御が開始されると、
先ず (I)開始増圧モードが実行される。(I)開始増圧モードは、所
定の増圧時間 T_{STA} の間、上記図 13 に示すアシスト圧増圧状態を
5 維持することにより実現される。上述の如く、アシスト圧増圧状態
が実現されると、各車輪のホイールシリンダ圧 $P_{w/c}$ は、フロントポン
プ 310 またはリアポンプ 312 を液圧源としてマスタシリンダ
圧 $P_{m/c}$ を超える圧力に昇圧される。従って、各車輪のホイールシリ
ンダ圧 $P_{w/c}$ は、BA制御の実行が開始された後、速やかにマスタ
10 シリンダ圧 $P_{m/c}$ を超える圧力に昇圧される。

上述した (I)開始増圧モードが終了すると、以後、運転者のブ
レーキ操作に対応して、(II)アシスト圧増圧モード、(III)アシス
ト圧減圧モード、(IV)アシスト圧保持モード、(V)アシスト圧緩増
モード、および、(VI)アシスト圧緩減モードの何れかが実行される。

15 BA制御の実行中に、マスタシリンダ圧 $P_{m/c}$ が急激に増圧され
ている場合は、運転者が更に大きな制動力を要求していると判断で
きる。本実施例の制動力制御装置では、この場合、(II)アシスト圧
増圧モードが実行される。(II)アシスト圧増圧モードは、上述した
(I)開始増圧モードと同様に、上記図 13 に示すアシスト圧増圧状
20 態を維持することにより実現される。アシスト圧増圧状態によれば、
各車輪のホイールシリンダ圧 $P_{w/c}$ を、フロントポンプ 310 および
リアポンプ 312 を液圧源として速やかに昇圧させることができる。
従って、上記の処理によれば、運転者の意図を正確にホイールシリ
ンダ圧 $P_{w/c}$ に反映させることができる。

25 BA制御の実行中に、マスタシリンダ圧 $P_{m/c}$ が急激に減圧され
ている場合は、運転者が制動力を速やかに低下させることを意図し
ていると判断できる。本実施例では、この場合、(III)アシスト圧
減圧モードが実行される。(III)アシスト圧減圧モードは、上記図
15 に示すアシスト圧減圧状態を維持することにより実現される。

アシスト圧減圧状態によれば、上述の如く、各車輪のホイールシリンダ圧 $P_{w/c}$ をマスタシリンダ圧 $P_{m/c}$ に向けて速やかに減圧させることができる。従って、上記の処理によれば、運転者の意図を正確にホイールシリンダ圧 $P_{w/c}$ に反映させることができる。

- 5 B A制御の実行中にマスタシリンダ圧 $P_{m/c}$ がほぼ一定値に維持されている場合は、運転者が制動力を保持することを意図していると判断できる。本実施例では、この場合、(IV)アシスト圧保持モードが実行される。(IV)アシスト圧保持モードは、上記図14に示すアシスト圧保持状態を維持することにより実現される。アシスト圧保持状態によれば、上述の如く、各車輪のホイールシリンダ圧 $P_{w/c}$ を一定値に維持することができる。従って、上記の処理によれば、運転者の意図を正確にホイールシリンダ圧 $P_{w/c}$ に反映させることができる。

- 15 B A制御の実行中にマスタシリンダ圧 $P_{m/c}$ が緩やかに増圧されている場合は、運転者が制動力を緩やかに立ち上げることを意図していると判断できる。本実施例では、この場合、(V)アシスト圧緩増モードが実行される。(V)アシスト圧緩増モードは、上記図13に示すアシスト圧増圧状態と上記図14に示すアシスト圧保持状態とを繰り返すことにより実現される。(V)アシスト圧緩増モードによれば、各車輪のホイールシリンダ圧 $P_{w/c}$ をフロントポンプ310およびリアポンプ312を液圧源として段階的に昇圧させることができる。従って、上記の処理によれば、運転者の意図を正確にホイールシリンダ圧 $P_{w/c}$ に反映させることができる。

- 25 B A制御の実行中にマスタシリンダ圧 $P_{m/c}$ が緩やかに減圧されている場合は、運転者が制動力を緩やかに低下させることを意図していると判断できる。本実施例では、この場合(VI)アシスト圧緩減モードが実行される。(VI)アシスト圧緩減モードは、上記図15に示すアシスト圧減圧状態と上記図14に示すアシスト圧保持状態とを繰り返すことにより実現される。(VI)アシスト圧緩減モードによ

れば、各車輪のホイールシリンダ圧 $P_{w/c}$ をマスタシリンダ圧 $P_{m/c}$ に向けて段階的に減圧させることができる。従って、上記の処理によれば、運転者の意図を正確にホイールシリンダ圧 $P_{w/c}$ に反映させることができる。

- 5 上記の処理によれば、運転者によって緊急ブレーキ操作が実行された後速やかに、ホイールシリンダ圧 $P_{w/c}$ をマスタシリンダ圧 $P_{m/c}$ に比して高い圧力に昇圧することができると共に、昇圧されたホイールシリンダ圧 $P_{w/c}$ を、運転者のブレーキ操作に応じて増減させることができる。
- 10 本実施例の制動力制御装置において、上述したBA制御が開始されると、その後、各車輪のホイールシリンダ圧 $P_{w/c}$ が速やかに昇圧されることにより、何れかの車輪について過剰なスリップ率が生ずる場合がある。ECU 210は、このような場合には、BA機能とABS機能とを共に実現するための制御（BA+ABS制御）を開始する。以下、上記図13乃至図15と共に図16乃至図21を参照して、BA+ABS制御の実行に伴う制動力制御装置の動作を説明する。

- 20 本実施例の制動力制御装置において、BA+ABS制御の実行中に、運転者によって制動力の減圧を意図するブレーキ操作が行われた場合は、ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ をABS制御の要求に応じた圧力に制御しつつ、ABS非対象車輪のホイールシリンダ圧 $P_{w/c}$ をマスタシリンダ圧 $P_{m/c}$ に向けて減圧する必要が生ずる。以下、この要求をアシスト圧減圧ABS要求と称す。

- 25 アシスト圧減圧ABS要求は、上記図15に示すアシスト圧減圧状態を実現しつつ、保持ソレノイド $S^{**}H$ および減圧ソレノイド $S^{**}R$ のうち、ABS対象車輪に対応するものをABS制御の要求に応じて適宜制御することで実現される。以下、制動力制御装置において上記の制御が実行されている状態をアシスト圧減圧ABS状態と称す。

アシスト圧減圧ABS要求は、運転者が制動力の減少を意図している場合に、すなわち、何れの車輪のホイールシリンダ圧 $P_{w/c}$ も増圧する必要がない場合に発生する。従って、アシスト圧減圧ABS要求が発生している状況下では、ABS非対象車輪のホイールシリンダ圧 $P_{w/c}$ を減圧しつつ、ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ を保持および減圧できることが必要である。

上述したアシスト圧減圧ABS状態においては、全ての保持ソレノイド $S^{**}H$ がマスタシリンダ218に連通している。このため、アシスト圧減圧ABS状態によれば、ABS非対象車輪のホイールシリンダ圧 $P_{w/c}$ を適正にマスタシリンダ圧 $P_{m/c}$ に向かって減圧することができる。また、かかる状況下でABS対象車輪について(i)保持モードまたは(iii)減圧モードが実現されると、ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ を保持または減圧することができる。このように、上述したアシスト圧減圧ABS状態によれば、アシスト圧減圧ABS要求が発生した際に実現すべき機能を、適切に実現することができる。

本実施例の制動力制御装置において、BA+ABS制御の実行中に運転者によって制動力の増加を意図するブレーキ操作が行われた場合は、ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ をABS制御の要求に応じた圧力に制御しつつ、ABS非対象車輪のホイールシリンダ圧 $P_{w/c}$ を、マスタシリンダ圧 $P_{m/c}$ を超える領域で増圧する必要が生ずる。以下、この要求をアシスト増圧ABS要求と称す。

アシスト圧増圧ABS要求は、上記図13に示すアシスト圧増圧状態を実現しつつ、保持ソレノイド $S^{**}H$ および減圧ソレノイド $S^{**}R$ のうちABS対象車輪に対応するものをABS制御の要求に応じて制御することによっても実現することができる。すなわち、例えば左前輪FLがABS対象車輪である場合に、上記図13に示すアシスト圧増圧状態を実現しつつSF_LH268およびSF_LR276をABS制御の要求に応じて制御すれば、左前輪FLのホイ

ルシリンダ圧 $P_{w/c}$ をABS制御の要求に応じた圧力に制御しつつ、他の車輪FR, RL, RRのオイルシリンダ圧 $P_{w/c}$ をマスタシリンダ圧 $P_{M/c}$ に比して高い領域で増圧することができる。

しかし、左前輪FLについてABS制御が開始されると、左前輪
5 FLに対応する保持ソレノイドSFLH268は、その後、左前輪FLについて(i)増圧モードが実行される僅かな時間を除き閉弁状態とされる。このため、左前輪FLについてABS制御が開始された後は、フロントポンプ310から吐出されるブレーキフルードの殆どが、ABS非対象車輪である右前輪FRのオイルシリンダ28
10 2に流入する。

フロントポンプ310の吐出能力は、左右前輪FL, FRのオイルシリンダ圧 $P_{w/c}$ を、同時に適当な増圧勾配で昇圧させることができるように設定されている。このため、フロントポンプ310から吐出されるブレーキフルードの殆どが、ABS非対象車輪である
15 右前輪FRのオイルシリンダ282に流入する状況下では、右前輪FRのオイルシリンダ圧 $P_{w/c}$ に過剰な増圧勾配が生ずる。

更に、上記の如く右前輪FRのオイルシリンダ圧 $P_{w/c}$ に過剰な増圧勾配が発生する状況下では、左前輪FLについて(i)増圧モードが実行された際に、左前輪FLのオイルシリンダ圧 $P_{w/c}$ が過度
20 に増圧される事態が生じ得る。ABS対象車輪のオイルシリンダ圧 $P_{w/c}$ が(i)増圧モードの実行に伴って過度に増圧されると、その車輪について再び(ii)減圧モードの実行が必要となり、ABS制御にハンチングが生じ易くなるという不都合が生ずる。

この点、上記図13に示すアシスト圧増圧状態を実現しつつ、保
25 持ソレノイドS**Hおよび減圧ソレノイドS**RのうちABS対象車輪に対応するものをABS制御の要求に応じて制御することによりアシスト圧増圧ABS要求を満たす手法は、本実施例の制動力制御装置においてBA+ABS制御を実現するための手法として必ずしも最適な手法ではない。

図 1 6 は、左前輪 F L を A B S 対象車輪とするアシスト圧増圧 A B S 要求が発生した場合に制動力制御装置において実現される状態（以下、アシスト圧増圧 A B S 状態と称す）の一形態を示す。左前輪 F L を A B S 対象車輪とするアシスト圧増圧 A B S 状態は、下記
5 (a) ～(d) の条件が満たされるように制動力制御装置を制御することにより実現される。

(a) 上記図 1 3 に示すアシスト圧増圧状態でオン状態とされているフロントリザーバカットソレノイド S R C F 2 3 0 をオフ状態とする。具体的には、(a-1) リアリザーバカットソレノイド S R C R
10 2 3 2、および、マスタカットソレノイド S M F R 2 4 2、S M F L 2 4 4、S M R 2 4 6 をオン状態とし、かつ、(a-2) フロントポンプ 3 1 0 およびリアポンプ 3 1 2 をオン状態とする。

(b) A B S 対象車輪である左前輪 F L の保持ソレノイド S F L H
15 2 6 8 および減圧ソレノイド S F L R 2 7 6 を A B S 制御の要求に応じて下記の如く制御する。(b-1) A B S 制御によって(ii)保持モードおよび(iii)減圧モードが要求される場合は、A B S 制御が単独で実行される場合と同様の手法により制御する。(b-2) A B S 制御によって(i)増圧モードの実行が要求される場合は、A B S 制御が単独で実行される場合に比して短縮された所定時間だけ増圧
20 モードを実行する。

(c) A B S 対象車輪と同一の系統に属する右前輪 F R の保持ソレノイド S F R H 2 6 6 を所定のデューティ比で繰り返しオン・オフさせる。

(d) A B S 対象車輪である左前輪 F L を含む系統に属するマスタ
25 カットソレノイド S M F R 2 4 2 および S M F L 2 4 4 を、左前輪 F L について(iii)減圧モードが実行される時期と同期してオフ状態（開弁状態）とする。

上記(a) の条件によれば、アシスト圧増圧 A B S 要求が生ずると同時に A B S 対象車輪を含む系統に属するフロントポンプ 3 1 0 と

リザーバタンク 224 とを遮断状態とすることができる。この場合、フロントポンプ 310 に吸入されるブレーキフルードがホイルシリンダ 284 から流出するフルードのみに限定されるため、フロントポンプ 310 の吐出側に発生する液圧が比較的低下に抑制される。

- 5 その結果、ABS制御のハンチングを防止するうえで、また、ABS非対象車輪である右前輪FRのホイルシリンダ圧 $P_{w/c}$ の増圧勾配を抑制するうえで有利な状態が形成される。

- 上記(b)の条件によれば、ABS対象車輪である左前輪FLで
(i) 増圧モードが実行される時間が、ABS制御が単独で実行され
10 る場合に比して短縮される。(i)増圧モードの実行時間が短縮されると、(i)増圧モードの実行に伴って左前輪FLのホイルシリンダ圧 $P_{w/c}$ に生ずる増圧量が抑制される。かかる状況下では、SFH 268の上流側に通常時に比して高圧の液圧が発生していても、ABS制御にハンチングは生じ難い。

- 15 上記(c)の条件によれば、ABS対象車輪と同一の系統に属する右前輪FRについて、ブレーキフルードがホイルシリンダ 282 に流入する状態と、その流入が阻止される状態とが所定のデューティ比で繰り返される。この場合、SFRH 266の上流側に通常時に比して高圧の液圧が発生していても、右前輪FRのホイルシリンダ
20 圧 $P_{w/c}$ は適正な増圧勾配で増圧する。

- 上記(d)の条件によれば、ホイルシリンダ 284 から流出したブレーキフルードがフロントポンプ 310 によって圧送される時期と同期して、フロントポンプ 310 の吐出側とマスタシリンダ 218 とが導通状態とされる。この場合、ブレーキフルードがマスタシリンダ 218 に流入し得るため、フロントポンプ 310 の吐出側に発生する液圧が比較的低下に抑制される。その結果、ABS制御のハンチングを防止するうえで、また、ABS非対象車輪である右前輪FRのホイルシリンダ圧 $P_{w/c}$ の増圧勾配を抑制するうえで有利な状態が形成される。
- 25

このため、上述したアシスト圧増圧ABS状態によれば、ABS対象車輪のオイルシリンダ圧 $P_{w/c}$ をABS制御が単独で実行される場合と同様に制御することができると共に、全てのABS非対象車輪のオイルシリンダ圧 $P_{w/c}$ を、BA制御が単独で実行されている状況下でオイルシリンダ圧 $P_{w/c}$ の増圧が要求された場合と同様の増圧勾配で増圧させることができる。このように、上述したアシスト圧増圧ABS状態によれば、アシスト圧増圧ABS要求が発生した際に実現すべき機能を、適切に実現することができる。

本実施例の制動力制御装置において、BA+ABS制御の実行中に、運転者によって制動力の保持を意図するブレーキ操作が行われた場合は、ABS対象車輪のオイルシリンダ圧 $P_{w/c}$ をABS制御の要求に応じた圧力に制御しつつ、ABS非対象車輪のオイルシリンダ圧 $P_{w/c}$ の保持を図る必要が生ずる。以下、この要求をアシスト圧保持ABS要求と称す。

アシスト圧保持ABS要求が生じた場合に、上記図14に示すアシスト圧保持状態を実現しつつ、保持ソレノイド $S^{**}H$ および減圧ソレノイド $S^{**}R$ のうちABS対象車輪に対応するものをABS制御の要求に応じて制御することによれば、ABS対象車輪のオイルシリンダ圧 $P_{w/c}$ をABS制御の要求に応じた圧力に制御すること、および、同一の系統内にABS対象車輪が含まれない系統に属するABS非対象車輪のオイルシリンダ圧 $P_{w/c}$ を保持することができる。

すなわち、例えば左前輪FLをABS対象車輪とするアシスト圧保持ABS要求が発生した場合に、上記図14に示すアシスト圧保持状態を実現しつつ $SFLH268$ および $SFLR276$ をABS制御の要求に応じて制御すれば、左前輪FLについては、(ii)保持モードおよび(iii)減圧モード、および、フロントポンプ310を液圧源とする(i)増圧モードを実現することができる。従って、左前輪FLのオイルシリンダ圧 $P_{w/c}$ は、ABS制御の要求に応じて

制御することができる。また、上記の状況下では、ABS対象車輪を含まない後輪の系統については、上記図14に示す状態と同様に維持される。従って、左右後輪RL, RRについては、BA制御が単独で実行される場合と同様に、それらのホイールシリンダ圧 $P_{w/c}$

5 を保持することができる。

しかし、上記の手法によると、左前輪FLについて (iii)減圧モードが実行された後、ホイールシリンダ284から流出したブレーキフルードがフロントポンプ310によって圧送され、右前輪FRのホイールシリンダ282に流入する。このため、同一の系統内にABS対象車輪を備える前輪の系統に属する右前輪FRについては、BA制御の要求に応えること、すなわち、ホイールシリンダ圧 $P_{w/c}$ を保持することができない。

図17は、左前輪FLをABS対象車輪とするアシスト圧保持ABS要求が発生した場合に制動力制御装置において実現される状態 (以下、アシスト圧保持ABS状態と称す) の一形態を示す。左前輪FLをABS対象車輪とするアシスト圧保持ABS状態は、下記 (e) ~ (g) の条件が満たされるように制動力制御装置を制御することにより実現される。

(e) 上記図14に示すアシスト圧保持状態でオフ状態とされている保持ソレノイドS**Hのうち、同一の系統内にABS対象車輪を有するABS非対象車輪である右前輪FRの保持ソレノイドSF RH 266をオン状態 (閉弁状態) とする。具体的には、(e-1) マスタカットソレノイドSMFR 242, SMFL 244, SMR 246をオン状態とし、(e-2) フロントポンプ310およびリアポンプ312をオン状態とし、かつ、(e-3) SF RH 266をオン状態とする。

(f) ABS対象車輪である左前輪FLの保持ソレノイドSFLH 268および減圧ソレノイドSFLR 276をABS制御の要求に応じて、上記(b) の条件と同様の手法で、すなわち、(i)増圧モー

ドの維持時間を通常時に比して短縮したパターンで制御する。

(g) ABS対象車輪である左前輪FLを含む系統に属するマスタ
カットソレノイドSMFR242およびSMFL244を、上記(c)
5 の条件と同様の手法で、すなわち、左前輪FLについて (iii)減
圧モードが実行される時期と同期してオフ状態（開弁状態）となる
ように制御する。

上記(e)の条件によれば、アシスト圧増圧ABS要求が生ずると
同時に、ABS対象車輪を含む系統に属するABS非対象車輪であ
る右前輪FRのオイルシリンダ282を、フロントポンプ310から
10 ら切り離すことができる。この場合、フロントポンプ310から吐
出されるブレーキフルードがオイルシリンダ282に流入しないた
め、右前輪FRのオイルシリンダ圧 $P_{w/c}$ がBA制御の要求に応じ
て適正に保持される。

上記(f)の条件によれば、上記(b)の条件が実現された場合と同
15 様に、ABS対象車輪である左前輪FLで (i)増圧モードが実行さ
れる際に、そのオイルシリンダ圧 $P_{w/c}$ に生ずる増圧量を抑制する
ことができる。

更に、上記(g)の条件によれば、上記(d)の条件が実現された場
合と同様に、オイルシリンダ284から流出したブレーキフルード
20 がフロントポンプ310によって圧送される時期と同期して、フロ
ントポンプ310の吐出側とマスタシリンダ218とを導通状態と
することができる。

従って、上述したアシスト圧保持ABS状態によれば、ABS対
象車輪のオイルシリンダ圧 $P_{w/c}$ をABS制御が単独で実行される
25 場合と同様に制御することができると共に、全てのABS非対象車
輪のオイルシリンダ圧 $P_{w/c}$ を、BA制御が単独で実行されている
場合と同様に適正に保持することができる。このように、上述した
アシスト圧保持ABS状態によれば、アシスト圧保持ABS要求が
発生した際に実現すべき機能を、適切に実現することができる。

本実施例の制動力制御装置は、BA制御が開始された後、何れかの車輪に過大なスリップ率が発生した場合に、適宜上述したアシスト圧増圧ABS状態、アシスト圧保持ABS状態、および、アシスト圧減圧ABS状態を実現することにより、①ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ をABS制御によって要求される圧力に抑制しつつ、②ABS非対象車輪のホイールシリンダ圧 $P_{w/c}$ をBA制御によって要求される圧力に制御する。

図18は、上述したBA制御とBA+ABS制御の双方を実現すべくECU210が実行するリザーバカットソレノイド制御ルーチンの一例のフローチャートを示す。ECU210は、左右前輪FL, FRが属する前輪系統、および、左右後輪RL, RRが属する後輪系統のそれぞれについて図18に示すルーチンを実行する。ECU210は、図18に示すルーチンを実行することで、リザーバカットソレノイドSRCF230およびSRCR232（以下、これらを総称する場合はリザーバカットソレノイドSRC*と称す）の状態を制御する。図18に示すルーチンは、所定時間毎に起動される定時割り込みルーチンである。図18に示すルーチンが起動されると、先ずステップ400の処理が実行される。

ステップ400では、制動力制御装置においてBA制御が実行されているか否かが判別される。その結果、BA制御が実行中でないと判別される場合は、以後、何ら処理が進められることなく今回のルーチンが終了される。一方、BA制御が実行中であると判別される場合は次にステップ402の処理が実行される。

ステップ402では、本ルーチンの制御対象とされている系統内に1輪以上ABS対象車輪が存在するか否かが判別される。その結果、1輪以上ABS対象車輪が存在すると判別された場合は、次にステップ404の処理が実行される。一方、制御対象とされている系統内にABS対象車輪が存在しないと判別された場合は、次にステップ406の処理が実行される。

ステップ 404では、リザーバカットソレノイドSRC*のうち、制御対象とされている系統に属するものがオフ状態（閉弁状態）とされる。本ステップ404の処理が終了すると、今回のルーチンが終了される。

- 5 ステップ406では、リザーバカットソレノイドSRC*のうち、制御対象とされている系統に属するものが、BA制御の要求に応じて通常通り制御される。本ステップ406の処理が終了すると、今回のルーチンが終了される。

- 10 上記図13乃至図15に示す如く、BA制御の実行中は、上記図13に示すアシスト圧増圧状態が要求される場合にリザーバカットソレノイドSRC*をオン状態（開弁状態）とする必要が生ずる。一方、上記図15乃至図17に示す如く、BA+ABS制御の実行中は、リザーバカットソレノイドSRC*のうち、ABS対象車輪が1輪も存在しない系統に属するものをBA制御中と同様に制御し、
15 かつ、少なくとも1輪のABS対象車輪を含む系統に属するものを常にオフ状態（閉弁状態）とする必要が生ずる。上記図18に示す制御ルーチンによれば、かかる要求を適切に満たすことができる。

- 20 また、上記図18に示す制御ルーチンによれば、BA制御の実行中にリザーバタンク224から流出するブレーキフルードの量を抑制することができる。BA制御の実行中にリザーバタンク224から多量のブレーキフルードが流出すると、マスタシリンダ218へ逆流するブレーキフルードの量が多量となり、逆止弁を構成するカップに損傷が生ずる、ブレーキペダル212が原位置に向けて不当に戻される等の不都合が生ずる。これに対して、上記図18に示す
25 制御ルーチンによれば、このような不都合が生ずるのを防止することができる。

図19は、上述したBA制御とBA+ABS制御の双方を実現すべくECU210が実行する制御手法選択ルーチンの一例のフローチャートを示す。ECU210は、各車輪毎に図19に示すルーチ

ンを実行する。ECU 210は、図19に示すルーチンを実行することで、保持ソレノイドS**Hおよび減圧ソレノイドS**Rの制御手法を各車輪毎に選択する。図19に示すルーチンは、所定時間毎に起動される定時割り込みルーチンである。図19に示すルーチンが起動されると、先ずステップ410の処理が実行される。

ステップ410では、制動力制御装置においてBA制御が実行されているか否かが判別される。その結果、BA制御が実行中でないと判別される場合は、以後、何ら処理が進められることなく今回のルーチンが終了される。一方、BA制御が実行中であると判別される場合は次にステップ412の処理が実行される。

ステップ412では、本ルーチンの制御対象とされている車輪（以下、この車輪を符号**を付して表す）がABS対象車輪であるか否かが判別される。その結果、制御対象車輪**がABS対象車輪であると判別された場合は、次にステップ414の処理が実行される。一方、制御対象車輪**がABS対象車輪でないと判別された場合は、次にステップ416の処理が実行される。

ステップ414では、制御対象車輪**に対応して設けられている保持ソレノイドS**Hおよび減圧ソレノイドS**Rの制御手法がABS制御に決定される。制御手法がABS制御とされたS**HおよびS**Rは、以後、制御対象車輪のスリップ状態に応じて適宜 (i)増圧モード、(ii)保持モードおよび (iii)減圧モードが実現されるように制御される。本ステップ414の処理が終了すると、今回のルーチンが終了される。

ステップ416では、制御対象車輪**と同一の系統に属する他の車輪がABS対象車輪であるか否かが判別される。その結果、他の車輪がABS対象車輪でないと判別された場合は、次にステップ418の処理が実行される。一方、他の車輪がABS対象車輪であると判別された場合は、次にステップ420の処理が実行される。

ステップ418では、制御対象車輪**に対応して設けられてい

る保持ソレノイドS**Hおよび減圧ソレノイドS**Rの制御手法がBA制御に決定される。本ステップ418で制御手法がBA制御とされたS**HおよびS**Rは、以後、BA制御の要求に応じて上記図13乃至図15に示す如く、具体的には常時オフ状態に、
5 制御される。本ステップ418の処理が終了すると、今回のルーチンが終了される。

ステップ420では、制御対象車輪**に対応して設けられている保持ソレノイドS**Hおよび減圧ソレノイドS**Rの制御手法がBA増圧勾配抑制制御に決定される。本ステップ420で制御
10 手法がBA制御とされたS**HおよびS**Rは、以後、BA+ABS制御の要求に応じて適宜制御される。

具体的には、BA+ABS制御によってアシスト圧増圧ABS要求が発生している場合は、減圧ソレノイドS**Rがオフ状態に維持されたまま、保持ソレノイドS**Hが所定のデューティ比でオン・オフされる。また、BA+ABS制御によってアシスト圧保持ABS要求が発生している場合は、保持ソレノイドS**Hがオン状態に、かつ、減圧ソレノイドS**Rがオフ状態に維持される。更に、BA+ABS制御によってアシスト圧減圧要求が発生している場合は、保持ソレノイドS**Hおよび減圧ソレノイドS**R
15 20 の双方がオフ状態に維持される。本ステップ420の処理が終了すると、今回のルーチンが終了される。

上記図13乃至図15に示す如く、BA制御が単独で実行されている場合、すなわち、前後何れの系統にもABS対象車輪が存在しない場合は、全ての保持ソレノイドS**Hおよび減圧ソレノイドS**Rを、常時オフ状態とする必要がある。また、上記図15乃至図17に示す如く、BA+ABS制御の実行中は、保持ソレノイドS**Hおよび減圧ソレノイドS**Rのうち、ABS対象車輪に対応して設けられたものをABSの要求に応じて制御し、ABS対象車輪が1輪も存在しない系統に属するABS非対象車輪に対応
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して設けられたものを常時オフ状態とし、かつ、ABS対象車輪と同一の系統に属するABS非対象車輪に対応して設けられたものを、アシスト圧増圧ABS要求が生じた際に上記(c)の条件が満たされるように、アシスト圧保持ABS要求が生じた際に上記(e)の条件が満たされるように、アシスト圧減圧ABS要求が生じた際にオフ状態となるように制御する必要がある。上記図19に示す制御ルーチンによれば、かかる要求を適切に満たすことができる。

図20は、BA+ABS制御の実行中に(i)増圧モードが実行された際にABS対象車輪のホイールシリンダ圧 $P_{w/c}$ に生ずる増圧量と、ABS制御が単独で実行されている場合に(i)増圧モードが実行された際にABS対象車輪のホイールシリンダ圧 $P_{w/c}$ に生ずる増圧量とをほぼ同量とするためにECU210が実行するABS制御手法選択ルーチンの一例のフローチャートを示す。

ECU210は、図20に示すルーチンを各車輪毎に実行する。

ECU210は、図20に示すルーチンを実行することで、ABS対象車輪に対応して設けられている保持ソレノイドS**Hおよび減圧ソレノイドS**Rを駆動する。図20に示すルーチンは、所定時間毎に起動される定時割り込みルーチンである。図20に示すルーチンが起動されると、先ずステップ430の処理が実行される。

ステップ430では、フラグXABS**に“1”がセットされているか否かが判別される。フラグXABS**は、本ルーチンの制御対象車輪**がABS対象車輪である場合に“1”とされるフラグである。従って、制御対象車輪**がABS対象車輪でない場合は、本ステップ430でXABS**=1が成立しないと判別される。この場合、次にステップ432の処理が実行される。

ステップ432では、制御対象車輪**についてABS制御の実行条件が成立したか否かが判別される。その結果、ABS制御の実行条件が成立していないと判別される場合は、以後、何ら処理が進められることなく今回のルーチンが終了される。一方、ABS制御

の実行条件が成立していると判別される場合は、次にステップ 4 3 4 の処理が実行される。

ステップ 4 3 4 では、制御対象車輪**がABS対象車輪となったことを表すべく、フラグXABS**に“1”がセットされる。

- 5 本ステップ 4 3 4 の処理が終了すると、次にステップ 4 3 6 の処理が実行される。

- ステップ 4 3 6 では、BA制御が実行中であるか否かが判別される。その結果、BA制御が実行中でない場合は、制御対象車輪**についてABS制御の実行条件が成立した後、ABS制御が単独で
10 実行されると判断することができる。この場合、次にステップ 4 3 8 の処理が実行される。一方、本ステップ 4 3 6 でBA制御が実行中であると判別される場合は、制御対象車輪**についてABS制御の実行条件が成立した後、BA+ABS制御が実行されると判断
15 することができる。この場合、次にステップ 4 4 0 の処理が実行される。

- ステップ 4 3 8 では、ABSマップに通常マップを設定する処理が実行される。ABSマップは、保持ソレノイドS**Hおよび減圧ソレノイドS**RをABS制御の要求に応じて駆動する際に参照されるマップである。本ステップ 4 3 8 でABSマップとされる
20 通常マップには、ABS制御が単独で実行される場合に、ABS対象車輪のオイルシリンダ圧 $P_{w/c}$ に適正な増圧勾配を発生させる駆動パターンが設定されている。本ステップ 4 3 8 の処理が終了すると、次にステップ 4 4 2 の処理が実行される。

- ステップ 4 4 0 では、ABSマップに増圧量抑制マップを設定する
25 処理が実行される。増圧量抑制マップは、BA+ABS制御の実行中に、ABS対象車輪のオイルシリンダ圧 $P_{w/c}$ に適正な増圧勾配を発生させる駆動パターン、すなわち、通常マップに比して(i)増圧モードの維持時間が短縮された駆動パターンが設定されている。本ステップ 4 4 0 の処理が終了すると、次にステップ 4 4 2 の処理

が実行される。

- 5 ステップ442では、上記ステップ438または440によって
 選択されたABSマップと、制御対象車輪**のスリップ状態とに
 基づいて保持ソレノイドS**Hおよび減圧ソレノイドS**Rが
 制御される。本ステップ442の処理が実行されることにより、A
 BS対象車輪について適宜 (i)増圧モード、(ii)保持モードおよび
 (iii)減圧モードが実現される。本ステップ442の処理が終了す
 ると、今回のルーチンが終了される。

- 10 上記ステップ430で、XSBS**=1が成立すると判別され
 る場合は、次にステップ444の処理が実行される。

- 15 ステップ444では、ABS制御の終了条件が成立しているか否
 かが判別される。その結果、ABS制御の終了条件が成立してい
 ないと判別される場合は、次に上述したステップ442の処理が実行
 される。ステップ442では、前回の処理サイクル時に以前に設定さ
 れたABSマップに従って、保持ソレノイドS**Hおよび減圧ソ
 レノイドS**Rが駆動される。一方、本ステップ444でABS
 制御の終了条件が成立していると判別される場合は、次にステップ
 446の処理が実行される。

- 20 ステップ446では、フラグXABS**を“0”とする処理が
 実行される。本ステップ446の処理が実行されると、以後、制御
 対象車輪**について再びABS制御の実行条件が成立するまで、
 その車輪**についてABS制御は実行されない。本ステップ44
 6の処理が終了すると、今回のルーチンが終了される。

- 25 上記の処理によれば、ABS制御が単独で実行される場合には、
 各車輪について通常マップに従った駆動パターンでABS制御を実
 行することができる。また、BA+ABS制御が実行される場合は、
 各車輪について増圧量抑制マップに従った駆動パターンでABS制
 御を実行することができる。このため、本実施例の制動力制御装置
 によれば、ABS制御が単独で実行される場合、および、BA+A

BS制御が実行される場合の双方において、制御上のハンチングを伴うことなくABS対象車輪のオイルシリンダ圧 $P_{w/c}$ を適正に制御することができる。

図21は、BA+ABS制御の実行中にABS対象車輪を含む系統に属するポンプの吐出側に不当に高い液圧が発生するのを防止すべくECU210が実行するマスタカットソレノイド制御ルーチンの一例のフローチャートを示す。ECU210は、図21に示すルーチンを前後輪の各系統毎に実行する。ECU210は、図21に示すルーチンを実行することで、ABS対象車輪を有する系統に属するマスタカットソレノイドSMFR242、SMFL244およびSMR246（以下、これらを総称する場合はマスタカットソレノイドSM**と称す）を駆動する。図21に示すルーチンは、所定時間毎に起動される定時割り込みルーチンである。図21に示すルーチンが起動されると、先ずステップ450の処理が実行される。

ステップ450では、BA制御が実行中であるか否かが判別される。その結果、BA制御が実行中であると判別される場合は、次にステップ452の処理が実行される。一方、BA制御が実行中でないと判別される場合は、次にステップ454の処理が実行される。

ステップ452では、本ルーチンの制御対象とされている系統に属するマスタカットソレノイドSM**をオフ状態（開弁状態）とする処理が実行される。本ステップ452の処理が終了すると、今回のルーチンが終了される。

ステップ454では、本ルーチンの制御対象とされている系統に、ABS制御の要求により(iii)減圧モードが実現されている車輪が存在するか否かが判別される。その結果、(iii)減圧モードが実現されている車輪が存在しないと判別された場合は、次にステップ456の処理が実行される。

ステップ456では、本ルーチンの制御対象とされている系統に

属するマスタカットソレノイドSM**がBA制御時と同様に制御される。具体的には、BA制御によってホイルシリンダ圧 $P_{w/c}$ の増圧または保持が要求されている場合はオン状態（閉弁状態）に、また、BA制御によってホイルシリンダ圧 $P_{w/c}$ の減圧が要求されている場合はオフ状態（開弁状態）に制御される（上記図13乃至図15のSM**および上記図16および図17のSMR246参照）。本ステップ456の処理が終了すると、今回のルーチンが終了される。

一方、上記ステップ454で、制御対象とされている系統に（ii）減圧モードとされている車輪が存在すると判別された場合は、次に上記ステップ452の処理、すなわち、その系統に属するマスタカットソレノイドSM**をオフ状態とする処理が実行される。

上記の処理によれば、BA+ABS制御の実行中、ABS対象車輪で（iii）減圧モードが実行される場合には、すなわち、ABS対象車輪と同一の系統に属するポンプがブレーキフルードの圧送を行う場合には、常にそのポンプの吐出側とマスタシリンダ218とが導通状態とされる。この場合、ポンプから吐出されたブレーキフルードがマスタシリンダ218に流入し得るため、ポンプから吐出されるブレーキフルードがABS対象車輪のホイルシリンダに流入することができないにも関わらず、ポンプの吐出側に不当に高い液圧が生ずることがない。このため、本実施例の制動力制御装置によれば、BA+ABS制御の実行中に、ABS対象車輪に制御上のハンチングを生じさせることがなく、かつ、ABS対象車輪と同一の系統に属するABS非対象車輪のホイルシリンダ圧 $P_{w/c}$ が過剰な増圧勾配で増圧されるのを確実に防止することができる。

ところで、上記の実施例においては、BA+ABS制御の実行中に、ABS対象車輪で（iii）減圧モードが実行される場合にのみ、その車輪と同一の系統に属するマスタカットソレノイドSM**を開弁状態とすることとしているが、本発明はこれに限定されるもの

ではなく、BA+ABS制御の実行中、常にマスタカットソレノイドSM**を開弁状態とすることとしてもよい。

尚、上記の実施例においては、マスタシリンダ218が「操作液圧発生手段」に、フロントポンプ310およびリアポンプ312が
5 「アシスト圧発生手段」に、液圧通路248, 250, 252が
「高圧通路」に、マスタカットソレノイドSM**が「操作液圧
カット機構」に、フロント減圧通路298およびリア減圧通路300が「低圧通路」に、保持ソレノイドS**Hおよび減圧ソレノイドS**Rが「導通状態制御機構」に、フロントリザーバ302お
10 よびリアリザーバ304が「低圧源」および「第2低圧源」に、また、リザーバタンク224が「第1低圧源」に、それぞれ相当している。

また、上記の実施例においては、ECU210が上記図20に示すルーチンを実行することにより「ABS制御手段」および「ABS
15 Sパターン選択手段」が、ECU210が上記図19に示すルーチンを実行することにより「BA増圧勾配抑制手段」が、ECU210が上記図18に示すルーチンを実行することにより「低圧源カット手段」が、それぞれ実現されている。

更に、上記の実施例においては、BA+ABS制御の実行中常に、
20 ECU210がマスタカットソレノイドSM**をオフ状態（開弁状態）とすることにより、「高圧通路開放手段」が実現される。

次に、図22乃至図27を参照して、本発明の第5実施例について説明する。図22は、本発明の第5実施例に対応するポンプアップ式制動力制御装置（以下、単に制動力制御装置と称す）のシステム構成図を示す。尚、図22において、上記図12に示す構成部分
25 と同一の部分については、同一の符号を付してその説明を省略または簡略する。

本実施例の制動力制御装置は、フロントエンジン・フロントドライブ式車両（FF車両）用の制動力制御装置として好適な装置であ

る。本実施例の制動力制御装置は、ECU 210により制御されている。ECU 210は、後述するリザーバカットソレノイドSRC₋₁およびSRC₋₂を上記ステップ404および406のSRC*として、また、後述するマスタカットソレノイドSMC₋₁512およびSMC₋₂514を上記ステップ452および456のSM**として上記図18乃至図21に示す制御ルーチンを実行することで、
5 上述した第4実施例の場合と同様に制動力制御装置の動作を制御する。

制動力制御装置は、ブレーキペダル212を備えている。ブレーキペダル212の近傍には、ブレーキスイッチ214が配設されている。ECU 210は、ブレーキスイッチ214の出力信号に基づいてブレーキペダル212が踏み込まれているか否かを判別する。

ブレーキペダル212は、バキュームブースタ216に連結されている。また、バキュームブースタ216は、マスタシリンダ218に固定されている。マスタシリンダ218の内部には第1油圧室220および第2油圧室222が形成されている。第1油圧室220および第2油圧室222の内部には、ブレーキ踏力Fと、バキュームブースタ216が発生するアシスト力F_aとの合力に応じたマスタシリンダ圧P_{m/c}が発生する。

マスタシリンダ218の上部にはリザーバタンク224が配設されている。リザーバタンク224には、第1リザーバ通路500、および、第2リザーバ通路502が連通している。第1リザーバ通路500には、第1リザーバカットソレノイド504（以下、SRC₋₁504と称す）が連通している。同様に、第2リザーバ通路502には、第2リザーバカットソレノイド506（以下、SRC₋₂506と称す）が連通している。

SRC₋₁504には、更に、第1ポンプ通路508が連通している。同様に、SRC₋₂506には、第2ポンプ通路510が連通している。SRC₋₁504は、オフ状態とされることで第1リザーバ

通路 5 0 0 と第 1 ポンプ通路 5 0 8 とを遮断し、かつ、オン状態とされることでそれらを導通させる 2 位置の電磁弁である。また、S R C -₂ 5 0 6 は、オフ状態とされることで第 2 リザーバ通路 5 0 2 と第 2 ポンプ通路 5 1 0 とを遮断し、かつ、オン状態とされることでそれらを導通させる 2 位置の電磁弁である。

5 マスタシリンダ 2 1 8 の第 1 油圧室 2 2 0、および、第 2 油圧室 2 2 2 には、それぞれ第 1 液圧通路 2 3 8、および、第 2 液圧通路 2 4 0 が連通している。第 1 液圧通路 2 3 8 には、第 1 マスタカットソレノイド 5 1 2（以下、S M C -₁ 5 1 2 と称す）が連通している。

10 一方、第 2 液圧通路 2 4 0 には、第 2 マスタカットソレノイド 5 1 4（以下、S M C -₂ 5 1 4 と称す）が連通している。

 S M C -₁ 5 1 2 には、第 1 ポンプ圧通路 5 1 6 と左後輪 R L に対応して設けられた液圧通路 5 1 8 とが連通している。第 1 ポンプ圧通路 5 1 6 には、第 1 ポンプソレノイド 5 2 0（以下、S M V -₁ 5 2 0 と称す）が連通している。S M V -₁ 5 2 0 には、更に、右前輪 F R に対応して設けられた液圧通路 5 2 2 が連通している。S M V -₁ 5 2 0 の内部には定圧開放弁 5 2 4 が設けられている。S M V -₁ 5 2 0 は、オフ状態とされた場合に第 1 ポンプ圧通路 5 1 6 と液圧通路 5 2 2 とを導通状態とし、かつ、オン状態とされた場合に定圧

15 開放弁 5 2 4 を介してそれらを連通させる 2 位置の電磁弁である。

 第 1 ポンプ圧通路 5 1 6 と液圧通路 5 2 2 との間には、また、第 1 ポンプ圧通路 5 1 6 側から液圧通路 5 2 2 側へ向かうフルードの流れのみを許容する逆止弁 5 2 6 が配設されている。

 S M C -₂ 5 1 4 には、第 2 ポンプ圧通路 5 2 8 と右後輪 R R に対応して設けられた液圧通路 5 3 0 とが連通している。第 2 ポンプ圧通路 5 2 8 には、第 2 ポンプソレノイド 5 3 2（以下、S M V -₂ 5 3 2 と称す）が連通している。S M V -₂ 5 3 2 には、更に、左前輪 F L に対応して設けられた液圧通路 5 3 4 が連通している。S M V -₂ 5 3 2 の内部には定圧開放弁 5 3 6 が設けられている。S M V -₂

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5 3 2は、オフ状態とされた場合に第2ポンプ圧通路5 2 8と液圧通路5 3 4とを導通状態とし、かつ、オン状態とされた場合に定圧開放弁5 3 6を介してそれらを連通させる2位置の電磁弁である。第1ポンプ圧通路5 2 8と液圧通路5 3 4との間には、また、第2
5 ポンプ圧通路5 2 8側から液圧通路5 3 6側へ向かうフルードの流れのみを許容する逆止弁5 3 8が配設されている。

SMC₋₁ 5 1 2およびSMC₋₂ 5 1 4の内部には、それぞれ定圧開放弁5 4 0, 5 4 2が設けられている。SMC₋₁ 5 1 2は、オフ
10 状態とされた場合に第1液圧通路2 3 8と液圧通路5 1 8（および第1ポンプ圧通路5 1 6）とを導通状態とし、かつ、オン状態とされた場合に定圧開放弁5 4 0を介してそれらを連通させる2位置の電磁弁である。また、SMC₋₂ 5 1 4は、オフ状態とされた場合に
15 第2液圧通路2 4 0と液圧通路5 3 0（および第2ポンプ圧通路5 2 8）とを導通状態とし、かつ、オン状態とされた場合に定圧開放弁5 4 2を介してそれらを連通させる2位置の電磁弁である。

第1液圧通路2 3 8と液圧通路5 1 8との間には、第1液圧通路2 3 8側から液圧通路5 1 8側へ向かうフルードの流れのみを許容する逆止弁5 4 4が配設されている。同様に、第2液圧通路2 4 0と液圧通路5 3 0との間には、第2液圧通路2 4 0側から液圧通路
20 5 3 0側へ向かう流体の流れのみを許容する逆止弁5 4 6が配設されている。

左右前輪および左右後輪に対応して設けられた4本の液圧通路5 1 6, 5 2 2, 5 2 8, 5 3 4には、第4実施例および第5実施例の場合と同様に保持ソレノイドS**H、減圧ソレノイドS**R、
25 ホイルシリンダ2 8 2～2 8 8および逆止弁2 9 0～2 9 6が連通している。また、右前輪FRおよび左後輪RLの減圧ソレノイドS FRR 2 7 4およびSRLR 2 8 0には、第1減圧通路5 4 8が連通している。更に、左前輪FLおよび右後輪RRの減圧ソレノイドS FLR 2 7 6およびS RRR 2 7 8には、第2減圧通路5 5 0が

連通している。

第1減圧通路548および第2減圧通路550には、それぞれ第1リザーバ552および第2リザーバ554が連通している。また、第1リザーバ552および第2リザーバ554は、それぞれ逆止弁556, 558を介して第1ポンプ560の吸入側、および、第2ポンプ562の吸入側に連通している。第1ポンプ560の吐出側、および、第2ポンプ562の吐出側は、吐出圧の脈動を吸収するためのダンパ564, 566に連通している。ダンパ564, 566は、それぞれ液圧通路522, 534に連通している。

10 各車輪の近傍には、車輪速センサ330, 332, 334, 336が配設されている。ECU210は、車輪速センサ330~336の出力信号に基づいて各車輪の回転速度 V_w を検出する。また、マスタシリンダ218に連通する第2液圧通路240には、液圧センサ338が配設されている。ECU210は、液圧センサ338
15 の出力信号に基づいてマスタシリンダ圧 P_{mc} を検出する。

次に、本実施例の制動力制御装置の動作を説明する。本実施例の制動力制御装置は、油圧回路内に配設された各種の電磁弁の状態を切り換えることにより、①通常ブレーキ機能、②ABS機能、および、③BA機能を実現する。

20 ①通常ブレーキ機能は、図22に示す如く、制動力制御装置が備える全ての電磁弁をオフ状態とすることにより実現される。以下、図22に示す状態を通常ブレーキ状態と称す。また、制動力制御装置において通常ブレーキ機能を実現するための制御を通常ブレーキ制御と称す。

25 図22に示す通常ブレーキ状態において、右前輪FRのホイルシリンダ282および左後輪RLのホイルシリンダ288は、共に第1液圧通路238を介してマスタシリンダ218の第1油圧室220に連通している。また、左前輪FLのホイルシリンダ284および右後輪RRのホイルシリンダ286は、共に第2液圧通路240

を介してマスタシリンダ 2 1 8 の第 2 油圧室 2 2 2 に連通している。
この場合、ホイルシリンダ 2 8 2 ~ 2 8 8 のホイルシリンダ圧 $P_{w/c}$ は、常にマスタシリンダ圧 $P_{m/c}$ と等圧に制御される。従って、
図 2 2 示す状態によれば、通常ブレーキ機能が実現される。

5 ② A B S 機能は、図 2 2 に示す状態において、第 1 ポンプ 5 6 0
および第 2 ポンプ 5 6 2 をオン状態とし、かつ、保持ソレノイド S
* * H および減圧ソレノイド S * * R を A B S の要求に応じて適当
に駆動することにより実現される。以下、制動力制御装置において
A B S 機能を実現するための制御を A B S 制御と称す。

10 A B S 制御の実行中は、左右前輪および左右後輪に対応して設け
られた 4 本の液圧通路 5 1 8, 5 2 2, 5 2 8, 5 3 4 の全てに高
圧のマスタシリンダ圧 $P_{m/c}$ が導かれている。従って、かかる状況
下で保持ソレノイド S * * H を開弁状態とし、かつ、減圧ソレノイ
ド S * * R を閉弁状態とすると、各車輪のホイルシリンダ圧 $P_{w/c}$
15 を増圧することができる。以下、この状態を (i) 増圧モードと称す。

また、A B S 制御の実行中に、保持ソレノイド S * * H および減
圧ソレノイド S * * R の双方を閉弁状態とすると、各車輪のホイル
シリンダ圧 $P_{w/c}$ を保持することができる。以下、この状態を (ii)
保持モードと称す。更に、A B S 制御の実行中に、保持ソレノイド
20 S * * H を閉弁状態とし、かつ、減圧ソレノイド S * * R を開弁状
態とすると、各車輪のホイルシリンダ圧 $P_{w/c}$ を減圧することがで
きる。以下、この状態を (iii) 減圧モードと称す。

E C U 2 1 0 は、A B S 制御の実行中に、各車輪毎に適宜上記の
(i) 増圧モード、(ii) 保持モード、および、(iii) 減圧モードが実
25 現されるように、各車輪のスリップ状態に応じて保持ソレノイド S
* * H および減圧ソレノイド S * * R を制御する。保持ソレノイド
S * * H および減圧ソレノイド S * * R が上記の如く制御されると、
全ての車輪のホイルシリンダ圧 $P_{w/c}$ が対応する車輪に過大なス
リップ率を発生させることのない適当な圧力に制御される。このよ

うに、上記の制御によれば、制動力制御装置においてABS機能を実現することができる。

- ABS制御の実行中に、各車輪で減圧モードが行われる際にはホイールシリンダ282～288内のブレーキフルードが、第1減圧通路548および第2減圧通路550を通過して第1リザーバ552および第2リザーバ554に流入する。第1リザーバ552および第2リザーバ554に流入したブレーキフルードは、第1ポンプ560および第2ポンプ562に汲み上げられて液圧通路522、534へ供給される。
- 10 液圧通路522、534に供給されたブレーキフルードの一部は、各車輪で(i)増圧モードが行われる際にホイールシリンダ282～288に流入する。また、そのブレーキフルードの残部は、ブレーキフルードの流出分を補うべくマスタシリンダ218に流入する。このため、本実施例のシステムによれば、ABS制御の実行中にブレーキペダル212に過大なストロークが生ずることはない。

- ③BA機能は、上記第4実施例の場合と同様に、運転者によって緊急ブレーキ操作が実行された後に、適宜(I)開始増圧モード、(II)アシスト圧増圧モード、(III)アシスト圧減圧モード、(IV)アシスト圧保持モード、(V)アシスト圧緩増モード、および、(VI)アシスト圧緩減モードが実現されるようにECU210が制動力制御装置を制御することにより実現される。以下、制動力制御装置において、BA機能を実現させるための制御をBA制御と称す。
- 20

- 図23は、BA制御の実行中に実現されるアシスト圧増圧状態を示す。アシスト圧増圧状態は、BA制御の実行中に各車輪のホイールシリンダ圧 $P_{w/c}$ を増圧させる必要がある場合に、すなわち、BA制御の実行中に(I)開始増圧モード、(II)アシスト圧増圧モード、および、(III)アシスト圧緩増モードの実行が要求された場合に実現される。
- 25

本実施例のシステムにおいて、BA制御中におけるアシスト圧増

圧状態は、図 2 3 に示す如く、リザーバカットソレノイド SRC -₁ 5 0 4, SRC -₂ 5 0 6、および、マスタカットソレノイド SMC -₁ 5 1 2, SMC -₂ 5 1 4 をオン状態とし、かつ、第 1 ポンプ 5 6 0 および第 2 ポンプ 5 6 2 をオン状態とすることで実現される。

- 5 BA 制御の実行中にアシスト圧増圧状態が実現されると、リザーバタンク 2 2 4 に貯留されているブレーキフルードが第 1 ポンプ 5 6 0 および第 2 ポンプ 5 6 2 に汲み上げられて液圧通路 5 2 2, 5 3 4 に供給される。アシスト圧増圧状態では、液圧通路 5 2 2 と右前輪 FR のホイルシリンダ 2 8 2 および左後輪 RL のホイルシリンダ 2 8 8 が導通状態に維持される。また、アシスト圧増圧状態では、
10 液圧通路 5 2 2 側の圧力が定圧開放弁 5 4 0 の開弁圧を超えてマスタシリンダ圧 $P_{m/c}$ に比して高圧となるまでは、液圧通路 5 2 2 側からマスタシリンダ 2 1 8 側へ向かうフルードの流れが SMC -₁ 5 1 2 によって阻止される。

- 15 同様に、アシスト圧増圧状態では、液圧通路 5 3 4 と左前輪 FL のホイルシリンダ 2 8 4 および右後輪 RR のホイルシリンダ 2 8 6 とが導通状態に維持されると共に、液圧通路 5 3 4 側の内圧が定圧開放弁 5 4 2 の開弁圧を超えてマスタシリンダ圧 $P_{m/c}$ に比して高圧となるまでは、液圧通路 5 3 4 側からマスタシリンダ 2 1 8 側へ
20 向かうフルードの流れが SMC -₂ 5 1 4 によって阻止される。

- このため、図 2 3 に示すアシスト圧増圧状態が実現されると、その後、各車輪のホイルシリンダ圧 $P_{w/c}$ は、第 1 ポンプ 5 6 0 または第 2 ポンプ 5 6 2 を液圧源として、速やかにマスタシリンダ圧 $P_{m/c}$ を超える圧力に昇圧される。このように、図 2 3 に示すアシスト圧増圧状態によれば、制動力を速やかに立ち上げることができる。
25

ところで、図 2 3 に示すアシスト圧増圧状態において、液圧通路 5 1 8, 5 2 2, 5 3 4, 5 3 0 は、逆止弁 5 4 4, 5 4 6 を介してマスタシリンダ 2 1 8 に連通している。このため、マスタシリンダ圧 $P_{m/c}$ が各車輪のホイルシリンダ圧 $P_{w/c}$ に比して大きい場合

は、BA作動状態においてもマスタシリンダ218を液圧源として
ホイールシリンダ圧 $P_{w/c}$ を昇圧することができる。

図24は、BA制御の実行中に実現されるアシスト圧保持状態を
示す。アシスト圧保持状態は、BA制御の実行中に各車輪のホイール
シリンダ圧 $P_{w/c}$ を保持する必要がある場合、すなわち、BA制御
中に(IV)アシスト圧保持モードが要求される場合に実現される。ア
シスト圧保持状態は、図24に示す如く、マスタカットソレノイド
SMC-₁512、SMC-₂514をオン状態とし、かつ、第1ポン
プ560および第2ポンプ562をオン状態とすることで実現され
る。

図24に示すアシスト圧保持状態では、第1ポンプ560とリ
ザーバタンク224、および、第2ポンプ562とリザーバタンク
224が、それぞれSRC-₁504およびSRC-₂506によって
遮断状態とされる。このため、アシスト圧保持状態では、第1ポン
プ560および第2ポンプ562から液圧通路522、534にフル
ードが吐出されない。また、図24に示すアシスト圧保持状態で
は、液圧通路518、522および530、534が、それぞれSMC-₁512
およびSMC-₂514によってマスタシリンダ218から実質的に切り離
されている。このため、図24に示すアシスト
圧保持状態によれば、全ての車輪のホイールシリンダ圧 $P_{w/c}$ を一定
値に保持することができる。

図25は、BA制御の実行中に実現されるアシスト圧減圧状態を
示す。アシスト圧減圧状態は、BA制御の実行中に各車輪のホイール
シリンダ圧 $P_{w/c}$ を減圧する必要がある場合、すなわち、BA制御
中に(III)アシスト圧減圧モード、および、(VI)アシスト圧緩減
モードの実行が要求された場合に実現される。アシスト圧減圧状態
は、図25に示す如く、第1ポンプ560および第2ポンプ562
をオン状態とすることで実現される。

図25に示すアシスト圧減圧状態では、第1ポンプ560および

第2ポンプ562がリザーバタンク224から切り離される。このため、第1ポンプ562および第2ポンプ562から液压通路522, 534にフルードが吐出されない。また、アシスト圧減圧状態では、各車輪のホイールシリンダ282~288とマスタシリンダ218とが導通状態となる。このため、アシスト圧減圧状態を実現すると、全ての車輪のホイールシリンダ圧 $P_{w/c}$ を、マスタシリンダ圧 $P_{m/c}$ を下限値として減圧することができる。

上述の如く、図23乃至図25に示すアシスト圧増圧状態、アシスト圧保持状態、および、アシスト圧減圧状態によれば、適切にBA制御の要求に応じてホイールシリンダ圧 $P_{w/c}$ の増圧、保持、および、減圧を図ることができる。このため、本実施例の制動力制御装置によっても、上述した第4実施例の場合と同様に、BA機能を実現することができる。

本実施例の制動力制御装置において、上述したBA制御が開始されると、その後、各車輪のホイールシリンダ圧 $P_{w/c}$ が速やかに昇圧されることにより、何れかの車輪について過剰なスリップ率が生ずる場合がある。ECU210は、このような場合には、BA+ABS制御を開始する。以下、上記図23乃至図25と共に図26および図27を参照して、BA+ABS制御の実行に伴う制動力制御装置の動作を説明する。

本実施例の制動力制御装置において、BA+ABS制御の実行中に、運転者によって制動力の減圧を意図するブレーキ操作が行われた場合は、ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ をABS制御の要求に応じた圧力に制御しつつ、ABS非対象車輪のホイールシリンダ圧 $P_{w/c}$ をマスタシリンダ圧 $P_{m/c}$ に向けて減圧する必要がある。以下、この要求をアシスト圧減圧ABS要求と称す。

アシスト圧減圧ABS要求は、上記図25に示すアシスト圧減圧状態を実現しつつ、保持ソレノイド S^{**H} および減圧ソレノイド S^{**R} のうち、ABS対象車輪に対応するものをABS制御の要

求に応じて適宜制御することで実現される。以下、制動力制御装置において上記の制御が実行されている状態をアシスト圧減圧ABS状態と称す。

5 アシスト圧減圧ABS要求は、運転者が制動力の減少を意図している場合に、すなわち、何れの車輪のホイールシリンダ圧 $P_{w/c}$ も増圧する必要がない場合に発生する。従って、アシスト圧減圧ABS要求が発生している状況下では、ABS非対象車輪のホイールシリンダ圧 $P_{w/c}$ を減圧しつつ、ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ を保持および減圧できることが必要である。

10 上述したアシスト圧減圧ABS状態においては、全ての保持ソレノイド $S^{**}H$ がマスタシリンダ218に連通している。このため、アシスト圧減圧ABS状態によれば、ABS非対象車輪のホイールシリンダ圧 $P_{w/c}$ を適正にマスタシリンダ圧 $P_{m/c}$ に向かって減圧することができる。また、かかる状況下でABS対象車輪について(i
15 i)保持モードまたは(iii)減圧モードが実現されると、ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ を保持または減圧することができる。このように、上述したアシスト圧減圧ABS状態によれば、アシスト圧減圧ABS要求が発生した際に実現すべき機能を、適切に実現することができる。

20 本実施例の制動力制御装置において、BA+ABS制御の実行中に運転者によって制動力の増加を意図するブレーキ操作が行われた場合は、ABS対象車輪のホイールシリンダ圧 $P_{w/c}$ をABS制御の要求に応じた圧力に制御しつつ、ABS非対象車輪のホイールシリンダ圧 $P_{w/c}$ を、マスタシリンダ圧 $P_{m/c}$ を超える領域で増圧する
25 必要が生ずる。以下、この要求をアシスト増圧ABS要求と称す。

アシスト増圧ABS要求は、上記図23に示すアシスト増圧状態を実現しつつ、保持ソレノイド $S^{**}H$ および減圧ソレノイド $S^{**}R$ のうちABS対象車輪に対応するものをABS制御の要求に応じて制御することによっても実現することができる。すなわち、

例えば左後輪RLがABS対象車輪である場合に、上記図23に示すアシスト圧増圧状態を実現しつつSR LH 272およびSR LR 280をABS制御の要求に応じて制御すれば、左後輪RLのオイルシリンダ圧 $P_{w/c}$ をABS制御の要求に応じた圧力に制御しつつ、
5 5 他車輪FL, FR, RLのオイルシリンダ圧 $P_{w/c}$ をマスタシリンダ圧 $P_{M/c}$ に比して高い領域で増圧することができる。

しかし、左後輪RLについてABS制御が開始されると、左後輪RLに対応する保持ソレノイドSR LH 272は、その後、左後輪RLについて (i)増圧モードが実行される僅かな時間を除き閉弁状態とされる。このため、左後輪RLについてABS制御が開始された後は、第1ポンプ560から吐出されるブレーキフルードの殆どが、ABS非対象車輪である右前輪FRのオイルシリンダ282に流入する。
10

第1ポンプ560の吐出能力は、右前輪FRのオイルシリンダ圧 $P_{w/c}$ と左後輪RLのオイルシリンダ圧 $P_{w/c}$ とを、同時に適当な増圧勾配で昇圧させることができるように設定されている。このため、第1ポンプ560から吐出されるブレーキフルードの殆どが、ABS非対象車輪である右前輪FRのオイルシリンダ282に流入する状況下では、右前輪FRのオイルシリンダ圧 $P_{w/c}$ に過剰な増圧勾配が生ずる。
15 20

更に、上記の如く右前輪FRのオイルシリンダ圧 $P_{w/c}$ に過剰な増圧勾配が発生する状況下では、左後輪RLについて (i)増圧モードが実行された際に、左後輪RLのオイルシリンダ圧 $P_{w/c}$ が過度に増圧される事態、すなわち、ABS制御にハンチングを生じさせ易い事態が形成される。
25

この点、上記図23に示すアシスト圧増圧状態を実現しつつ、保持ソレノイドS**Hおよび減圧ソレノイドS**RのうちABS対象車輪に対応するものをABS制御の要求に応じて制御することによりアシスト圧増圧ABS要求を満たす手法は、本実施例の制動

力制御装置においてB A + A B S制御を実現するための手法として必ずしも最適な手法ではない。

図 2 6 は、左後輪 R L を A B S 対象車輪とするアシスト圧増圧 A B S 要求が発生した場合に制動力制御装置において実現される状態
5 (以下、アシスト圧増圧 A B S 状態と称す) の一形態を示す。左後輪 R L を A B S 対象車輪とするアシスト圧増圧 A B S 状態は、下記 (a) ~ (d) の条件が満たされるように制動力制御装置を制御することにより実現される。

(a) 上記図 2 3 に示すアシスト圧増圧状態でオン状態とされている第 1 リザーバカットソレノイド S R C -₁ 5 0 4 をオフ状態とする。
10 具体的には、(a-1) 第 2 リザーバカットソレノイド S R C -₂ 5 0 6、および、マスタカットソレノイド S M C -₁ 5 1 2, S M C -₂ 5 1 4 をオン状態とし、かつ、(a-2) フロントポンプ 3 1 0 およびリアポンプ 3 1 2 をオン状態とする。

15 (b) A B S 対象車輪である左後輪 R L の保持ソレノイド S R L H 2 7 2 および減圧ソレノイド S R L R 2 8 0 を A B S 制御の要求に応じて下記の如く制御する。(b-1) A B S 制御によって(ii)保持モードおよび (iii)減圧モードが要求される場合は、A B S 制御が単独で実行される場合と同様の手法により制御する。(b-2) A B S
20 制御によって (i)増圧モードの実行が要求される場合は、A B S 制御が単独で実行される場合に比して短縮された所定時間だけ増圧モードを実行する。

(c) A B S 対象車輪と同一の系統に属する右前輪 F R の保持ソレノイド S F R H 2 6 6 を所定のデューティ比で繰り返しオン・オフ
25 させる。

(d) A B S 対象車輪である左後輪 R L を含む系統に属するマスタカットソレノイド S M C -₁ 5 1 2 を、左後輪 R L について (iii)減圧モードが実行される時期と同期してオフ状態(開弁状態)とする。

上記(a)の条件によれば、アシスト圧増圧 A B S 要求が生ずると

同時にABS対象車輪を含む系統に属する第1ポンプ560とリ
ザーバタンク224とを遮断状態とすることができる。この場合、
第1ポンプ560に吸入されるブレーキフルードがホイルシリンダ
288から流出するフルードのみに限定されるため、第1ポンプ5
5 60の吐出側に発生する液圧が比較的低下に抑制される。その結果、
ABS制御のハンチングを防止するうえで、また、ABS非対象車
輪である右前輪FRのホイルシリンダ圧 $P_{w/c}$ の増圧勾配を抑制す
るうえで有利な状態が形成される。

上記(b)の条件によれば、ABS対象車輪である左後輪RLで
10 (i) 増圧モードが実行される時間が、ABS制御が単独で実行され
る場合に比して短縮される。(i)増圧モードの実行時間が短縮され
ると、(i)増圧モードの実行に伴って左後輪RLのホイルシリンダ
圧 $P_{w/c}$ に生ずる増圧量が抑制される。かかる状況下では、SR
LH272の上流側に通常時に比して高圧の液圧が発生していても、
15 ABS制御にハンチングは生じ難い。

上記(c)の条件によれば、ABS対象車輪と同一の系統に属する
右前輪FRについて、ブレーキフルードがホイルシリンダ282に
流入する状態と、その流入が阻止される状態とが所定のデューティ
比で繰り返される。この場合、SFRH266の上流側に通常時に
20 比して高圧の液圧が発生していても、右前輪FRのホイルシリンダ
圧 $P_{w/c}$ は適正な増圧勾配で増圧する。

上記(d)の条件によれば、ホイルシリンダ288から流出したブ
レーキフルードが第1ポンプ560によって圧送される時期と同期
して、第1ポンプ560の吐出側とマスタシリンダ218とが導通
25 状態とされる。この場合、ブレーキフルードがマスタシリンダ21
8に流入し得るため、第1ポンプ560の吐出側に発生する液圧が
比較的低下に抑制される。その結果、ABS制御のハンチングを防
止するうえで、また、ABS非対象車輪である右前輪FRのホイル
シリンダ圧 $P_{w/c}$ の増圧勾配を抑制するうえで有利な状態が形成さ

れる。

このため、上述したアシスト圧増圧ABS状態によれば、ABS
対象車輪のオイルシリンダ圧 $P_{w/c}$ をABS制御が単独で実行され
る場合と同様に制御することができると共に、全てのABS非対象
5 車輪のオイルシリンダ圧 $P_{w/c}$ を、BA制御が単独で実行されてい
る状況下でオイルシリンダ圧 $P_{w/c}$ の増圧が要求された場合と同様
の増圧勾配で増圧させることができる。このように、上述したアシ
スト圧増圧ABS状態によれば、アシスト圧増圧ABS要求が発生
した際に実現すべき機能を、適切に実現することができる。

10 本実施例の制動力制御装置において、BA+ABS制御の実行中
に、運転者によって制動力の保持を意図するブレーキ操作が行われ
た場合は、ABS対象車輪のオイルシリンダ圧 $P_{w/c}$ をABS制御
の要求に応じた圧力に制御しつつ、ABS非対象車輪のオイルシリ
ンダ圧 $P_{w/c}$ の保持を図る必要が生ずる。以下、この要求をアシ
15 ト圧保持ABS要求と称す。

アシスト圧保持ABS要求が生じた場合に、上記図24に示すア
シスト圧保持状態を実現しつつ、保持ソレノイド $S^{**}H$ および減
圧ソレノイド $S^{**}R$ のうちABS対象車輪に対応するものをABS
S制御の要求に応じて制御することによれば、ABS対象車輪のホ
20 イルシリンダ圧 $P_{w/c}$ をABS制御の要求に応じた圧力に制御する
こと、および、同一の系統内にABS対象車輪が含まれない系統に
属するABS非対象車輪のオイルシリンダ圧 $P_{w/c}$ を保持すること
ができる。

すなわち、例えば左後輪RLをABS対象車輪とするアシスト圧
25 保持ABS要求が発生した場合に、上記図24に示すアシスト圧保
持状態を実現しつつ $SRLH272$ および $SRLR280$ をABS
制御の要求に応じて制御すれば、左後輪RLについては、(ii)保持
モードおよび(iii)減圧モード、および、第1ポンプ560を液圧
源とする(i)増圧モードを実現することができる。従って、左後輪

RLのホイルシリンダ圧 $P_{w/c}$ は、ABS制御の要求に応じて制御
することができる。また、上記の状況下では、ABS対象車輪を含
まない後輪の系統については、上記図24に示す状態と同様に維持
される。従って、左前輪FLおよび右後輪RRについては、BA制
5 御が単独で実行される場合と同様に、それらのホイルシリンダ圧 $P_{w/c}$
を保持することができる。

しかし、上記の手法によると、左後輪RLについて(iii)減圧
モードが実行された後、ホイルシリンダ288から流出したブレー
キフルードが第1ポンプ560にによって圧送され、右前輪FRの
10 ホイルシリンダ282に流入する。このため、同一の系統内にABS
対象車輪を備える前輪の系統に属する右前輪FRについては、BA
制御の要求に応えること、すなわち、ホイルシリンダ圧 $P_{w/c}$ を
保持することができない。

図27は、左後輪RLをABS対象車輪とするアシスト圧保持A
15 BS要求が発生した場合に制動力制御装置において実現される状態
(以下、アシスト圧保持ABS状態と称す)の一形態を示す。左後
輪RLをABS対象車輪とするアシスト圧保持ABS状態は、下記
(e)~(g)の条件が満たされるように制動力制御装置を制御するこ
とにより実現される。

20 (e) 上記図24に示すアシスト圧保持状態でオフ状態とされてい
る保持ソレノイドS**Hのうち、同一の系統内にABS対象車輪
を有するABS非対象車輪である右前輪FRの保持ソレノイドSFRH
266をオン状態(閉弁状態)とする。具体的には、(e-1)マ
スタカットソレノイドSMC-1512、SMC-2514をオン状態
25 とし、(e-2)第1ポンプ560および第2ポンプ562をオン状態
とし、かつ、(e-3)SFRH266をオン状態とする。

(f) ABS対象車輪である左後輪RLの保持ソレノイドSRLH
272および減圧ソレノイドSRLR280をABS制御の要求に
応じて、上記(b)の条件と同様の手法で、すなわち、(i)増圧モー

ドの維持時間を通常時に比して短縮したパターンで制御する。

(g) A B S対象車輪である左後輪 R Lを含む系統に属する第 1 マスタカットソレノイド S M C - 1 5 1 2 を、上記(c) の条件と同様の手法で、すなわち、左後輪 R Lについて (iii)減圧モードが実行される時期と同期してオフ状態（開弁状態）となるように制御する。

上記(e) の条件によれば、アシスト圧増圧 A B S 要求が生ずると同時に、A B S対象車輪を含む系統に属する A B S非対象車輪である右前輪 F Rのオイルシリンダ 2 8 2 を、第 1 ポンプ 5 6 0 から切り離すことができる。この場合、第 1 ポンプ 5 6 0 から吐出されるブレーキフルードがオイルシリンダ 2 8 2 に流入しないため、右前輪 F Rのオイルシリンダ圧 $P_{w/c}$ が B A制御の要求に応じて適正に保持される。

上記(f) の条件によれば、上記(b) の条件が実現された場合と同様に、A B S対象車輪である左後輪 R Lで (i)増圧モードが実行される際に、そのオイルシリンダ圧 $P_{w/c}$ に生ずる増圧量を抑制することができる。

更に、上記(g) の条件によれば、上記(d) の条件が実現された場合と同様に、オイルシリンダ 2 8 8 から流出したブレーキフルードが第 1 ポンプ 5 6 0 によって圧送される時期と同期して、第 1 ポンプ 5 6 0 の吐出側とマスタシリンダ 2 1 8 とを導通状態とすることができる。

従って、上述したアシスト圧保持 A B S 状態によれば、A B S対象車輪のオイルシリンダ圧 $P_{w/c}$ を A B S制御が単独で実行される場合と同様に制御することができると共に、全ての A B S非対象車輪のオイルシリンダ圧 $P_{w/c}$ を、B A制御が単独で実行されている場合と同様に適正に保持することができる。このように、上述したアシスト圧保持 A B S 状態によれば、アシスト圧保持 A B S 要求が発生した際に実現すべき機能を、適切に実現することができる。

本実施例の制動力制御装置によれば、A B S制御が単独で実行さ

れている場合、B A制御が単独で実行されている場合、および、B A + A B S制御が実行されている場合のそれぞれに対応して、適宜上記図 2 2 乃至図 2 7 に示す状態が実現される。このため、本実施例の制動力制御装置によれば、A B S制御またはB A制御が単独で
5 実行されている場合に、ホイルシリンダ圧 $P_{w/c}$ をそれらの要求に応じた適切な液圧に制御することができると共に、B A + A B S制御が実行されている場合に、① A B S対象車輪のホイルシリンダ圧 $P_{w/c}$ をA B S制御によって要求される圧力に、また、② A B S非対象車輪のホイルシリンダ圧 $P_{w/c}$ をB A制御によって要求される
10 圧力に、それぞれ精度良く制御することができる。

ところで、上記の実施例においては、B A + A B S制御の実行中に、A B S対象車輪で (iii)減圧モードが実行される場合にのみ、その車輪と同一の系統に属するマスタカットソレノイド SMC-₁ 5 1 2, SMC-₂ 5 1 4 開弁状態とすることとしているが、本発明は
15 これに限定されるものではなく、B A + A B S制御の実行中、常にそれらを開弁状態とすることとしてもよい。

尚、上記の実施例においては、第 1 ポンプ 5 6 0 および第 2 ポンプ 5 6 2 が「アシスト圧発生手段」に、液圧通路 5 1 8, 5 2 2, 5 3 0, 5 3 4 が「高圧通路」に、第 1 マスタカットソレノイド SMC-₁ 5 1 2 および第 2 マスタカットソレノイド SMC-₂ 5 1 4 が
20 「操作液圧カット機構」に、第 1 減圧通路 5 4 8 および第 2 減圧通路 5 5 0 が「低圧通路」に、また、第 1 リザーバ 5 5 2 および第 2 リザーバ 5 5 4 が「低圧源」および「第 2 低圧源」に、それぞれ相当している。

25

請求の範囲

1. ホイルシリンダに連通する液圧流入経路を遮断した状態でホイルシリンダ圧を制御する制動液圧減圧制御と、運転者によって緊急ブレーキ操作が実行された際に通常時に比して大きな制動液圧を発生させるブレーキアシスト制御とを実行する制動力制御装置において、

- 5 ホイルシリンダの液圧流入経路（56, 62）の導通状態を検出する導通検出手段（ステップ104）と、
- 10 前記ブレーキアシスト制御の開始時に、何れかのホイルシリンダの液圧流入経路が実質的に遮断されている場合には、他のホイルシリンダへの制動液圧の流入を抑制する液圧流入抑制手段（ステップ108）と、
- を備えることを特徴とする制動力制御装置。

- 15
2. 車輪のスリップ状態に関する特性値が所定のしきい値を超える場合に、該車輪のホイルシリンダに連通する液圧流入経路を遮断した状態でホイルシリンダ圧を減圧する減圧制御を実行した後に、該ホイルシリンダについて所定の液圧制御を実行する制動液圧制御と、運転者によって緊急ブレーキ操作が実行された際に通常時に比して大きな制動液圧を発生させるブレーキアシスト制御とを実行する制動力制御装置において、

- 20 ホイルシリンダの液圧流入経路（56, 62）の導通状態を検出する導通検出手段（ステップ112）と、
- 25 何れかのホイルシリンダの液圧流入経路が実質的に遮断された状態で前記ブレーキアシスト制御が開始された場合には、前記少なくとも他の一のホイルシリンダについて前記減圧制御が実行されることにより生ずる減圧傾向を、通常時に比して強める減圧傾向変更手段（ステップ118）と、

を備えることを特徴とする制動力制御装置。

3. 請求項2記載の制動力制御装置において、

- 5 何れかのホイルシリンダの液圧流入経路(56、62)が実質的に遮断された状態で前記ブレーキアシスト制御が開始された際に、前記少なくとも他の一のホイルシリンダについての前記しきい値を通常時に比して小さな値にするしきい値変更手段(ステップ118)を備えることを特徴とする制動力制御装置。

- 10 4. 運転者によって緊急ブレーキ操作が行われた際に通常時に比して大きな制動油圧を発生させるブレーキアシスト制御と、各車輪の制動油圧を各車輪に過剰なスリップ率を発生させない圧力に制御するアンチロックブレーキ制御と、を実行する制動力制御装置において、

- 15 ブレーキ操作量に応じた制動液圧を発生する操作液圧発生手段(218)と、

ブレーキ操作量と無関係に所定の制動液圧を発生するアシスト圧発生手段(310、312; 560, 562)と、

- 20 前記操作液圧発生手段および前記アシスト圧発生手段の双方に連通する高圧通路(248, 250, 252; 518, 522, 530, 534)と、

前記操作液圧発生手段と前記高圧通路とを遮断状態とし得る操作液圧カット機構(242, 244; 512, 514)と、

- 25 所定の低圧源(302, 304; 552, 554)に連通する低圧通路(298, 300; 548, 550)と、

各車輪のホイルシリンダと前記高圧通路との導通状態、および、各車輪のホイルシリンダと前記低圧通路との導通状態を制御する導通状態制御機構(266-280)と、

運転者によって緊急ブレーキ操作が行われた場合に、前記操作液

圧カット機構を遮断状態とし、かつ、前記アシスト圧発生手段から前記高圧通路に所定の制動液圧を供給させるB A制御手段と、

- 前記導通状態制御機構を所定の制御パターンで制御することにより、各車輪に過剰なスリップ率が生じないように各車輪のホイールシリンダ圧を制御するABS制御手段（FIG. 20のルーチン）と、
- 5 アンチロックブレーキ制御が単独で実行されている場合に前記制御パターンを通常パターンとし、アンチロックブレーキ制御とブレーキアシスト制御とが同時に実行されている場合に、前記制御パターンをホイールシリンダ圧の増圧量を抑制するための増圧量抑制パターンとするABS制御パターン選択手段（FIG. 20のルーチン）と、
- 10 を備えることを特徴とする制動力制御装置。

5. 運転者によって緊急ブレーキ操作が行われた際に通常時に比
- 15 して大きな制動油圧を発生させるブレーキアシスト制御と、各車輪の制動油圧を各車輪に過剰なスリップ率を発生させない圧力に制御するアンチロックブレーキ制御と、を実行する制動力制御装置において、

- ブレーキ操作量に応じた制動液圧を発生する操作液圧発生手段
- 20 （218）と、

ブレーキ操作量と無関係に所定の制動液圧を発生するアシスト圧発生手段（310、312；560、562）と、

- 前記操作液圧発生手段および前記アシスト圧発生手段の双方に連通する高圧通路（248，250，252；518，522，530，534）と、
- 25

前記操作液圧発生手段と前記高圧通路とを遮断状態とし得る操作液圧カット機構（242，244；512，514）と、

所定の低圧源（302，304；552，554）に連通する低圧通路（298，300；548，550）と、

各車輪のオイルシリンダと前記高圧通路との導通状態、および、各車輪のオイルシリンダと前記低圧通路との導通状態を制御する導通状態制御機構（266-280）と、

- 5 運転者によって緊急ブレーキ操作が行われた場合に、前記操作液圧カット機構を遮断状態とし、かつ、前記アシスト圧発生手段から前記高圧通路に所定の制動液圧を供給させるBA制御手段と、

- 前記導通状態制御機構を所定の制御パターンで制御することにより、各車輪に過剰なスリップ率が生じないように各車輪のオイルシリンダ圧を制御するABS制御手段（FIG. 20のルーチン）と、
10 ブレーキアシスト制御とアンチロックブレーキ制御とが同時に実行されている場合に、アンチロックブレーキ制御の非対象車輪のオイルシリンダ圧の増圧勾配が抑制されるように、前記非対象車輪に対応して設けられている前記導通状態制御機構を制御するBA増圧勾配抑制手段（FIG. 19のルーチン）と、
15 を備えることを特徴とする制動力制御装置。

6. 運転者によって緊急ブレーキ操作が行われた際に通常時に比して大きな制動油圧を発生させるブレーキアシスト制御と、各車輪の制動油圧を各車輪に過剰なスリップ率を発生させない圧力に制御
20 するアンチロックブレーキ制御と、を実行する制動力制御装置において、

ブレーキ操作量に応じた制動液圧を発生する操作液圧発生手段（218）と、

- 第1低圧源（224）および第2低圧源（302, 304; 55
25 2, 554）に連通する低圧通路（298, 300; 548, 550）と、

前記低圧通路から吸入したブレーキフルードを圧送することによりブレーキ操作量と無関係に所定の制動液圧を発生するアシスト圧発生手段（310, 312; 560, 562）と、

前記操作液圧発生手段および前記アシスト圧発生手段の双方に連通する高压通路（２４８，２５０，２５２；５１８，５２２，５３０，５３４）と、

5 前記操作液圧発生手段と前記高压通路とを遮断状態とし得る操作液圧カット機構（２４２，２４４；５１２，５１４）と、

各車輪のホイールシリンダと前記高压通路との導通状態、および、各車輪のホイールシリンダと前記低压通路との導通状態を制御する導通状態制御機構（２６６－２８０）と、

10 運転者によって緊急ブレーキ操作が行われた場合に、前記操作液圧カット機構を遮断状態とし、かつ、前記アシスト圧発生手段から前記高压通路に所定の制動液圧を供給させるＢＡ制御手段と、

前記導通状態制御機構を所定の制御パターンで制御することにより、各車輪に過剰なスリップ率が生じないように各車輪のホイールシリンダ圧を制御するＡＢＳ制御手段（ＦＩＧ．２０のルーチン）と、
15 ブレーキアシスト制御とアンチロックブレーキ制御とが同時に実行されている場合に、前記第１低压源と前記アシスト圧発生手段とを遮断状態とする低压源カット手段（ＦＩＧ．１８のルーチン）と、
を備えることを特徴とする制動力制御装置。

20 ７．運転者によって緊急ブレーキ操作が行われた際に通常時に比して大きな制動油圧を発生させるブレーキアシスト制御と、各車輪の制動油圧を各車輪に過剰なスリップ率を発生させない圧力に制御するアンチロックブレーキ制御と、を実行する制動力制御装置において、

25 ブレーキ操作量に応じた制動液圧を発生する操作液圧発生手段（２１８）と、

ブレーキ操作量と無関係に所定の制動液圧を発生するアシスト圧発生手段（３１０，３１２；５６０，５６２）と、

前記操作液圧発生手段および前記アシスト圧発生手段の双方に連

通する高圧通路（２４８，２５０，２５２；５１８，５２２，５３０，５３４）と、

前記操作液圧発生手段と前記高圧通路とを遮断状態とし得る操作液圧カット機構（２４２，２４４；５１２，５１４）と、

- 5 所定の低圧源（３０２，３０４；５５２，５５４）に連通する低圧通路（２９８，３００；５４８，５５０）と、

各車輪のホイルシリンダと前記高圧通路との導通状態、および、各車輪のホイルシリンダと前記低圧通路との導通状態を制御する導通状態制御機構（２６６－２８０）と、

- 10 運転者によって緊急ブレーキ操作が行われた場合に、前記操作液圧カット機構を遮断状態とし、かつ、前記アシスト圧発生手段から前記高圧通路に所定の制動液圧を供給させるＢＡ制御手段と、

前記導通状態制御機構を所定の制御パターンで制御することにより、各車輪に過剰なスリップ率が生じないように各車輪のホイルシ

- 15 リンダ圧を制御するＡＢＳ制御手段（ＦＩＧ．２０のルーチン）と、
ブレーキアシスト制御とアンチロックブレーキ制御とが同時に実行されている場合に、前記操作液圧カット機構を導通状態とする高圧通路開放手段と、

を備えることを特徴とする制動力制御装置。

20

８．請求項６記載の制動力制御装置において、

ブレーキアシスト制御とアンチロックブレーキ制御とが同時に実行されており、かつ、アンチロックブレーキ制御の対象車輪においてホイルシリンダ圧の減圧が図られている場合に、前記操作液圧

- 25 カット機構を導通状態とする高圧通路開放手段を備えることを特徴とする制動力制御装置。

FIG. 2

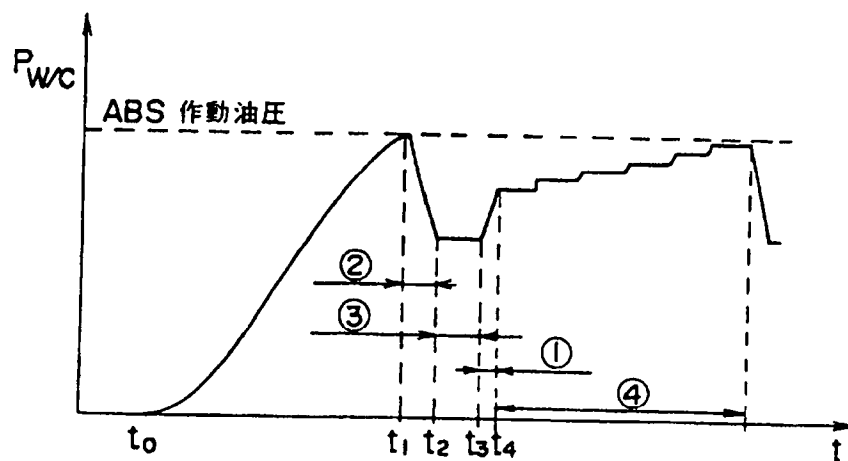


FIG. 3

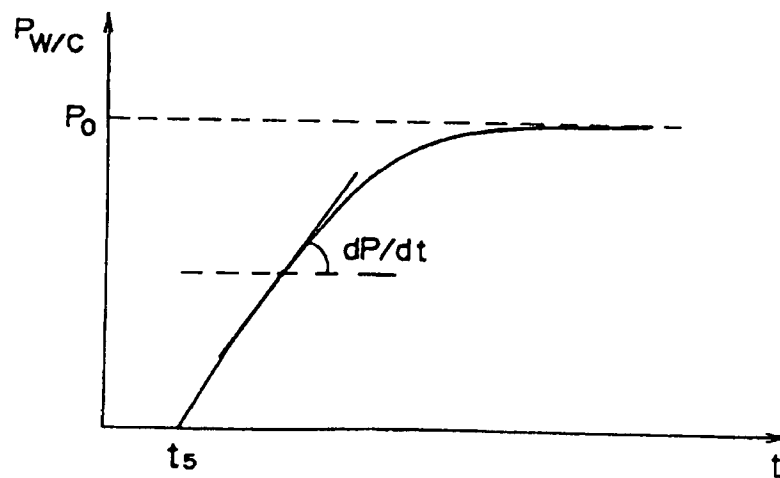


FIG. 4

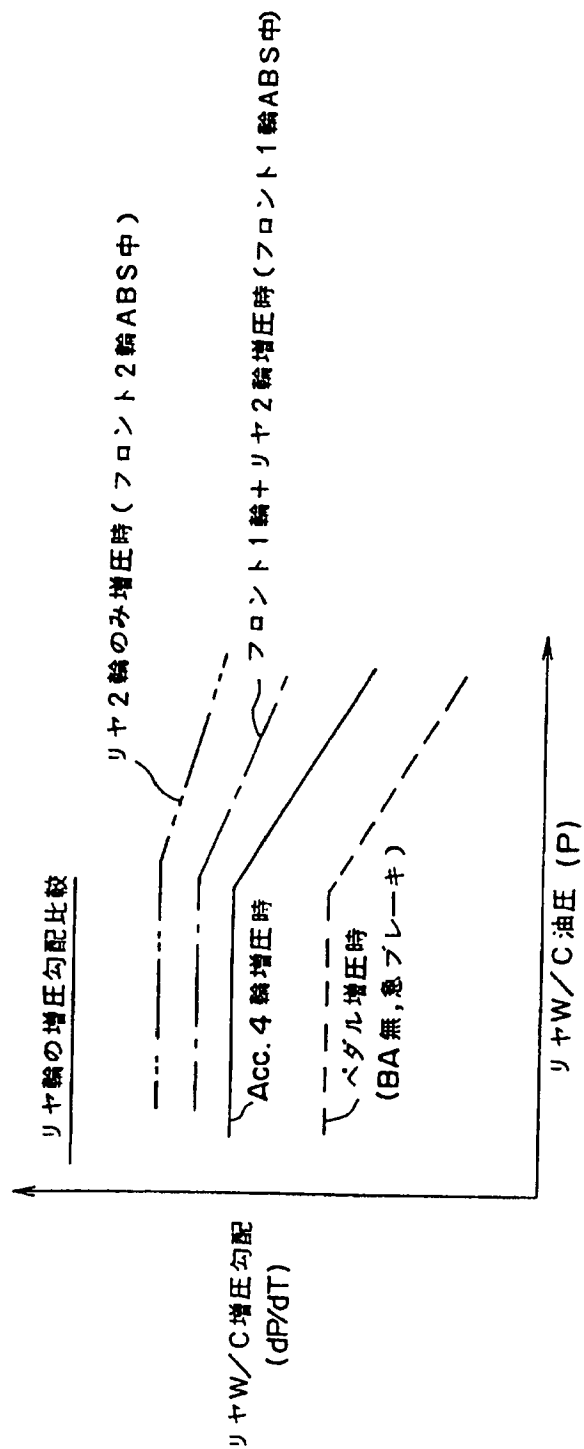


FIG. 5

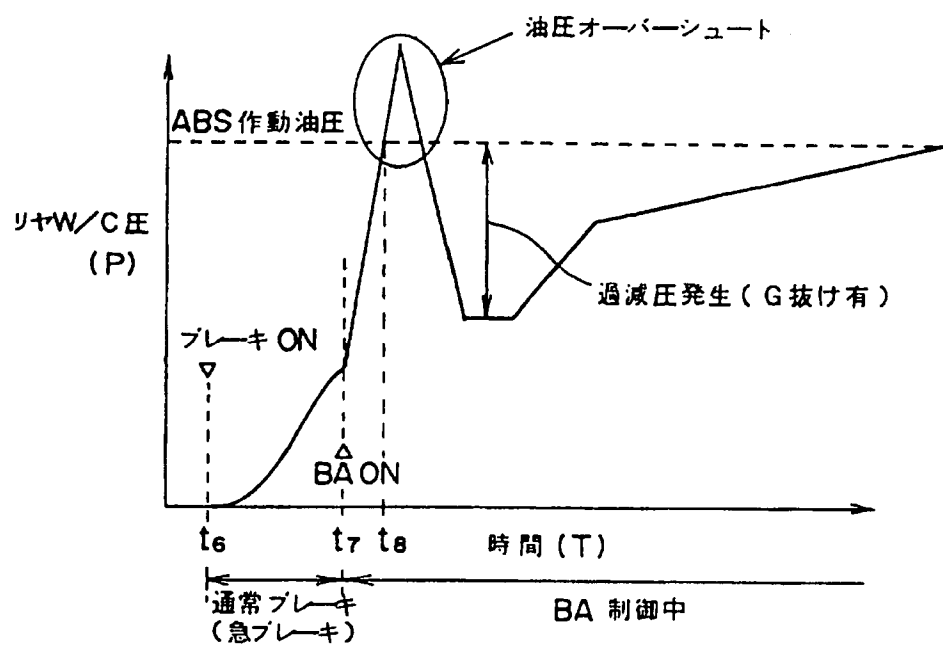


FIG. 6

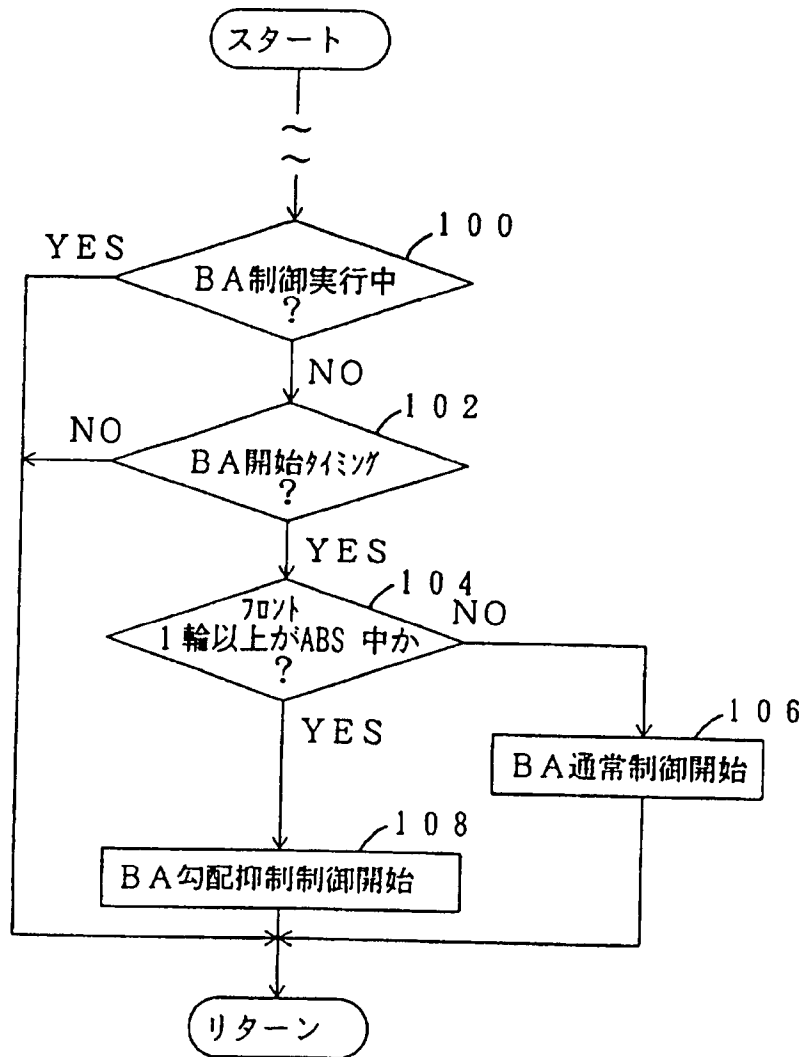


FIG. 7

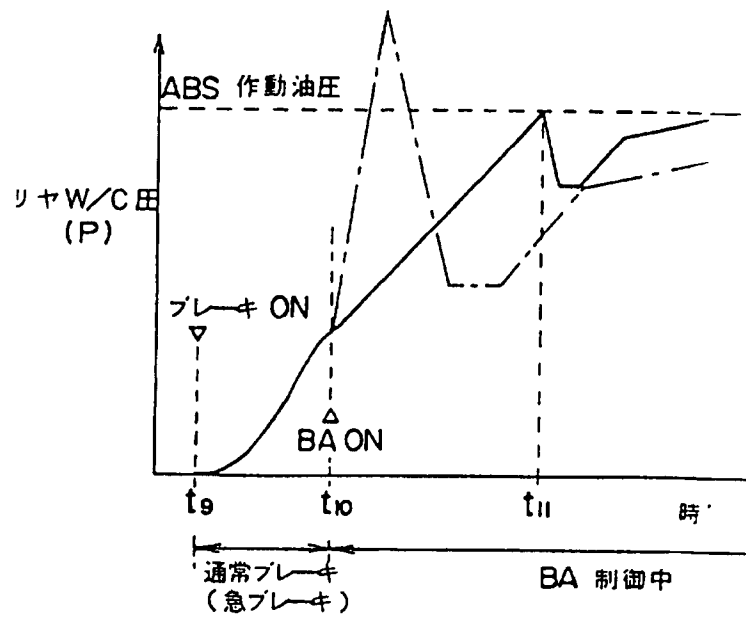


FIG. 8

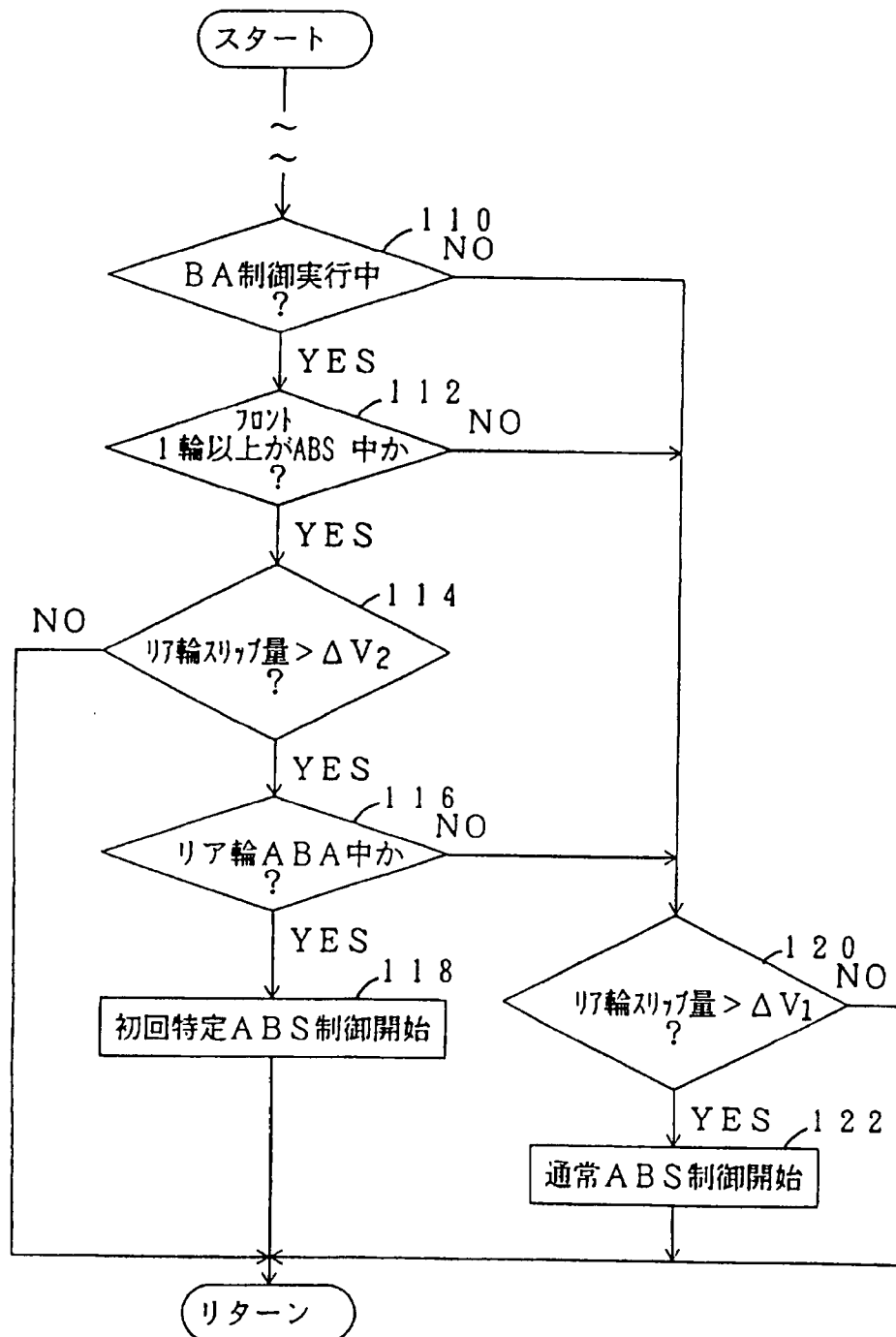


FIG. 9

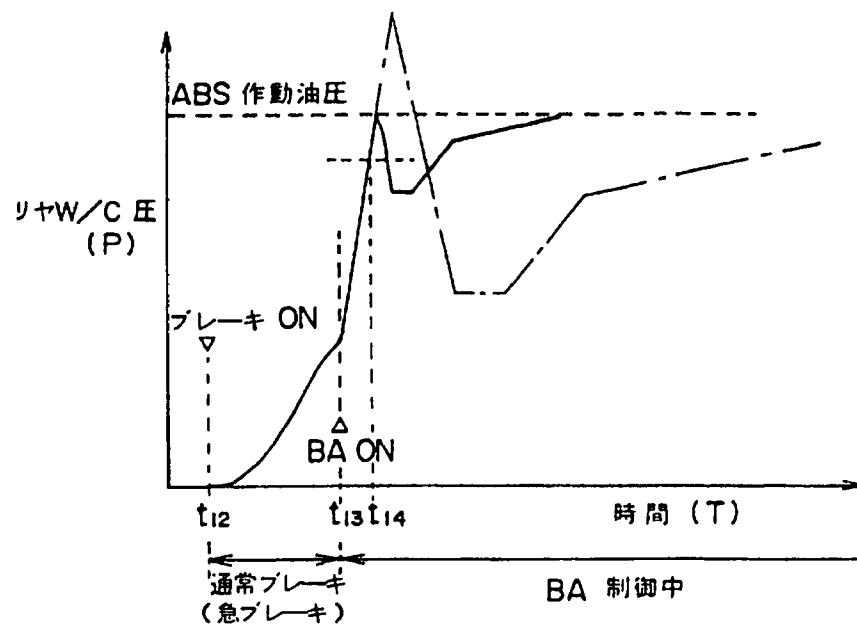


FIG. 10

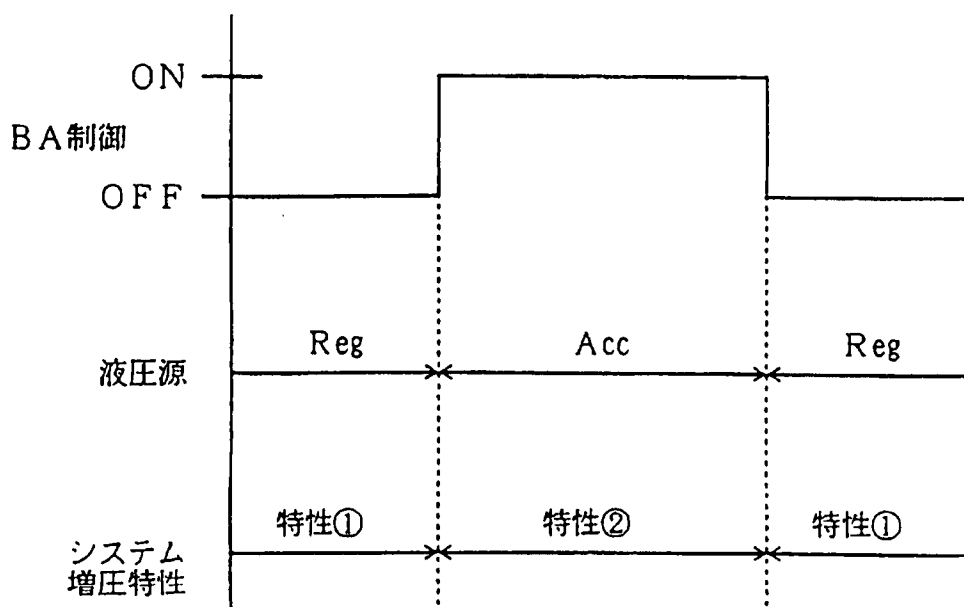


FIG. 11

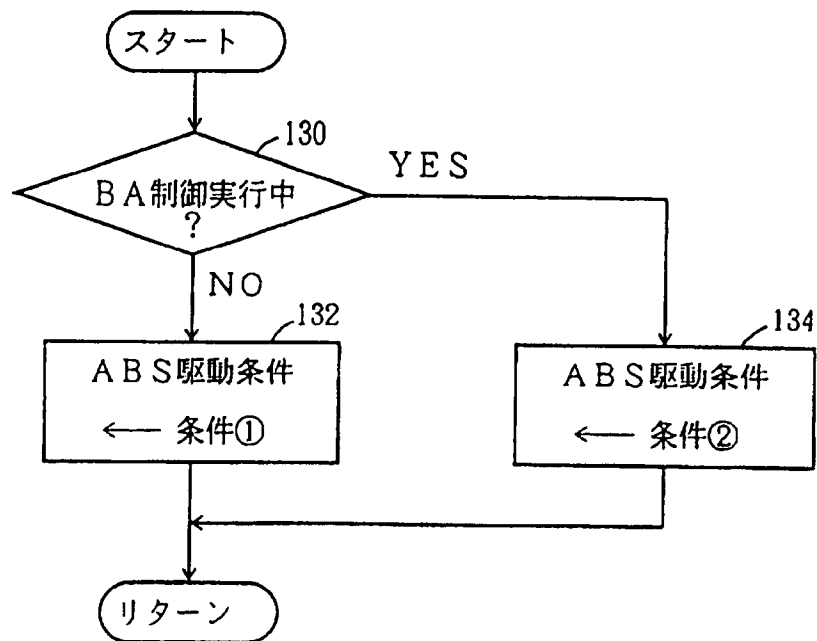


FIG. 12

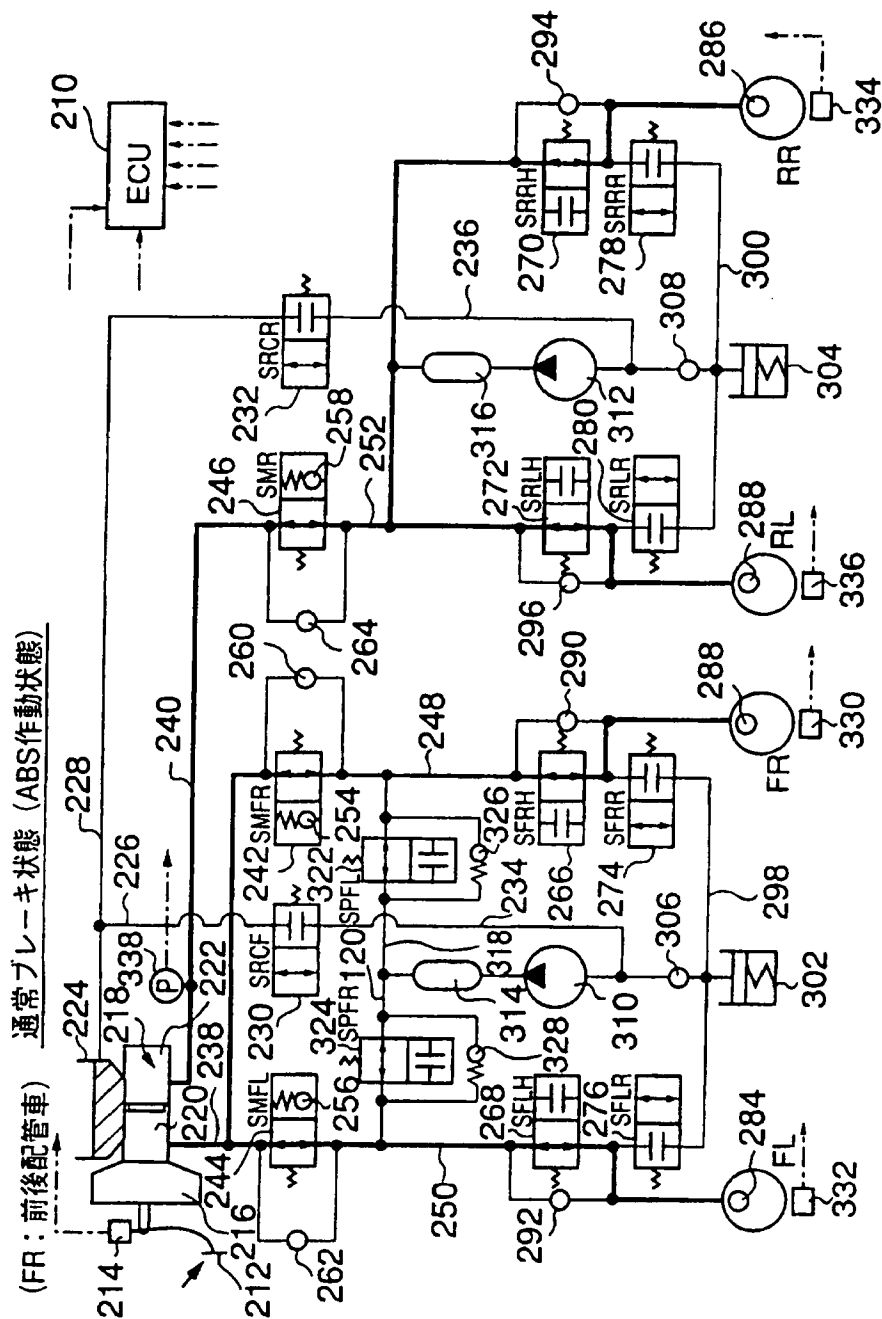


FIG. 13

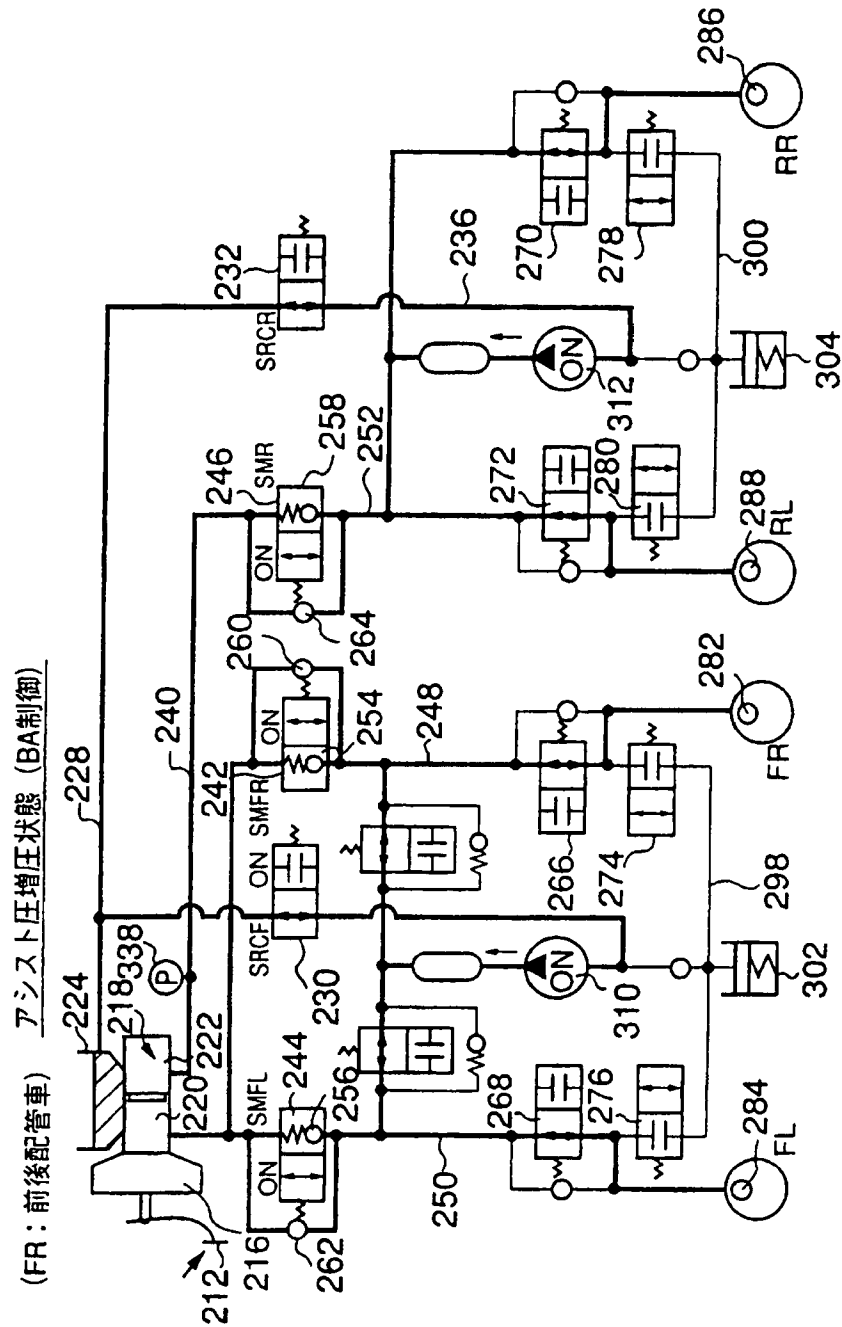


FIG. 14

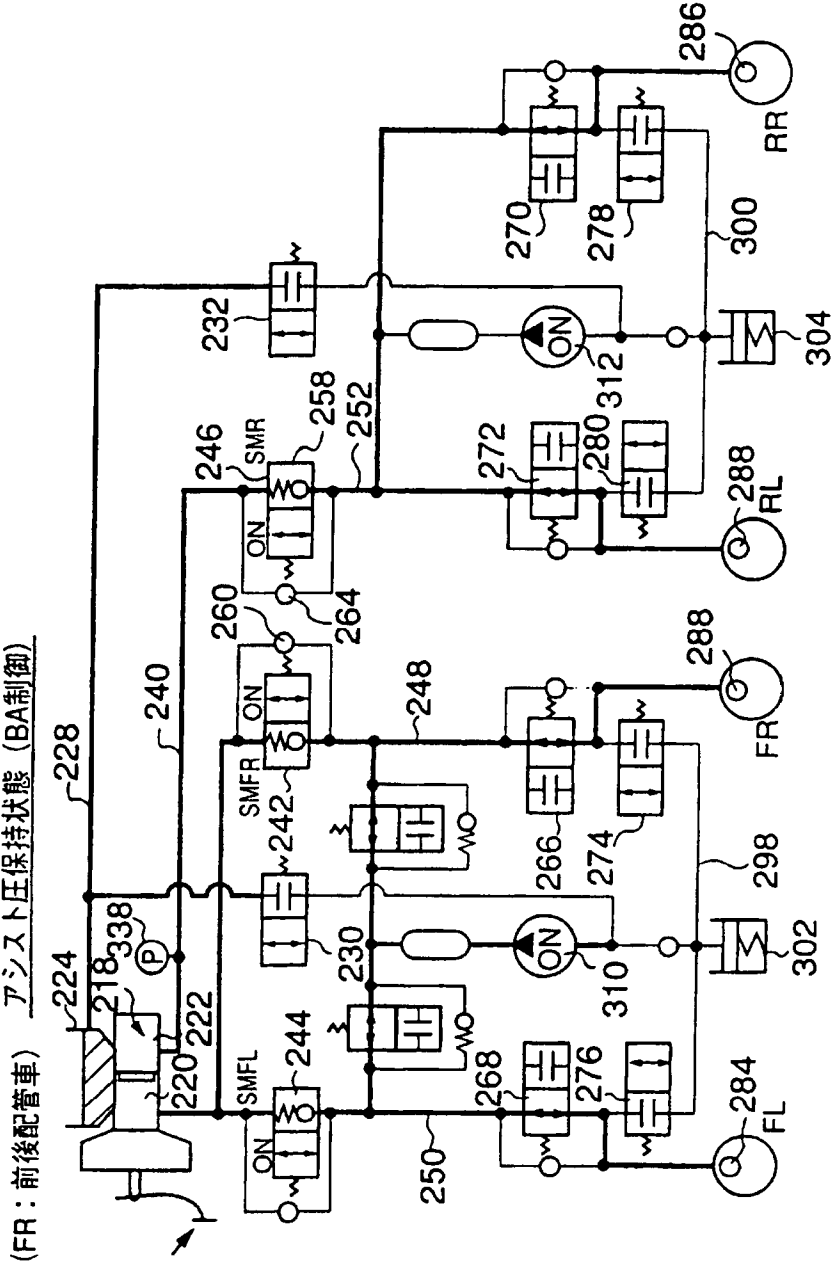


FIG. 15

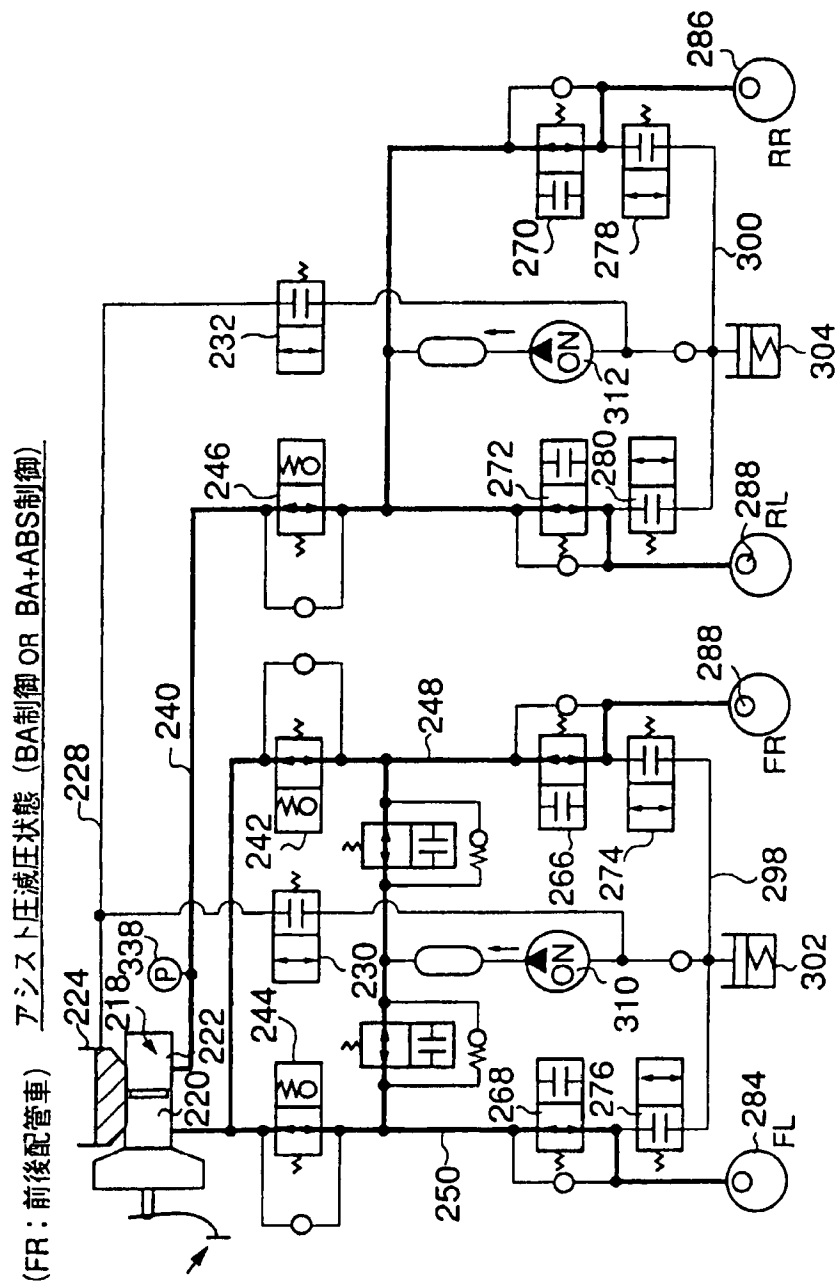


FIG. 16

アシスト圧増圧状態(BA+ABS制御)

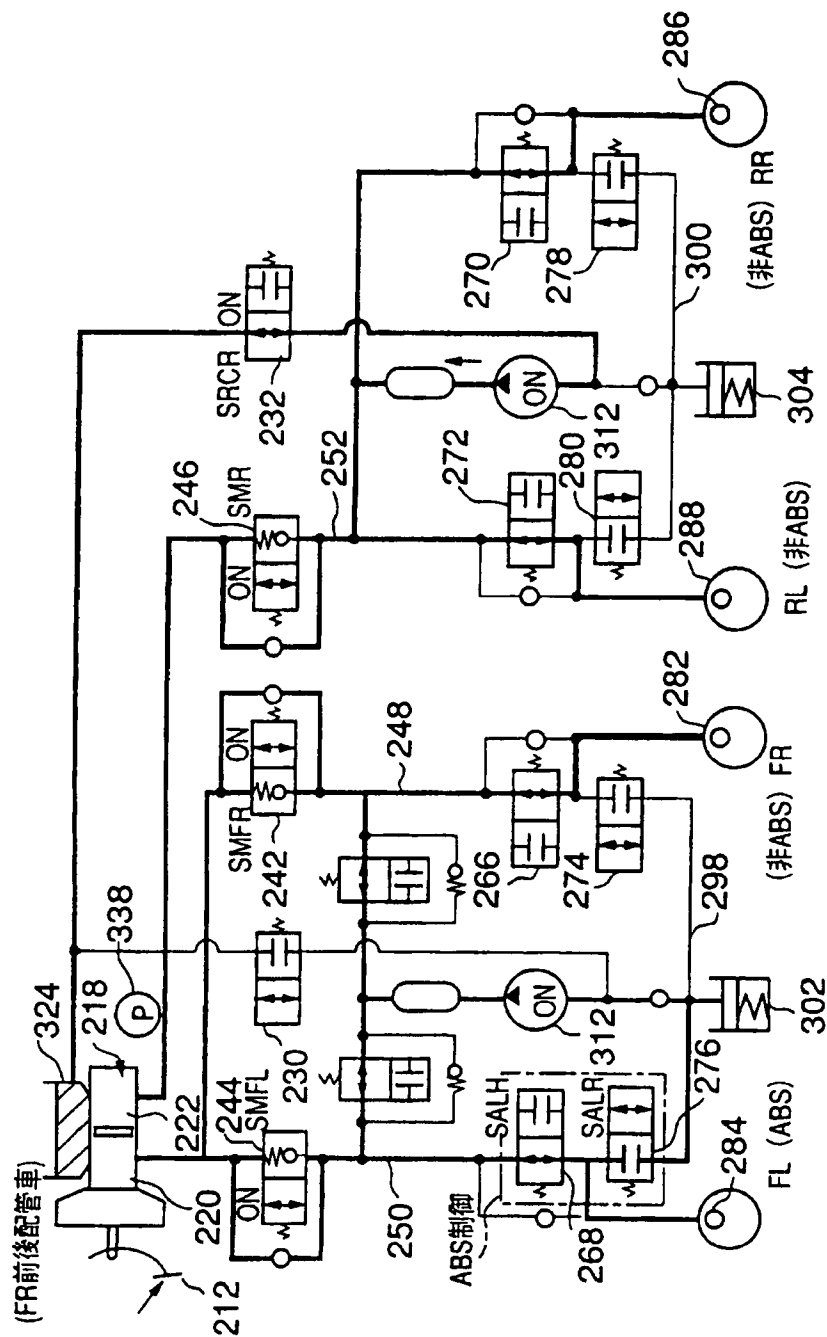


FIG. 17

アシスト圧保持状態(BA+ABS制御)

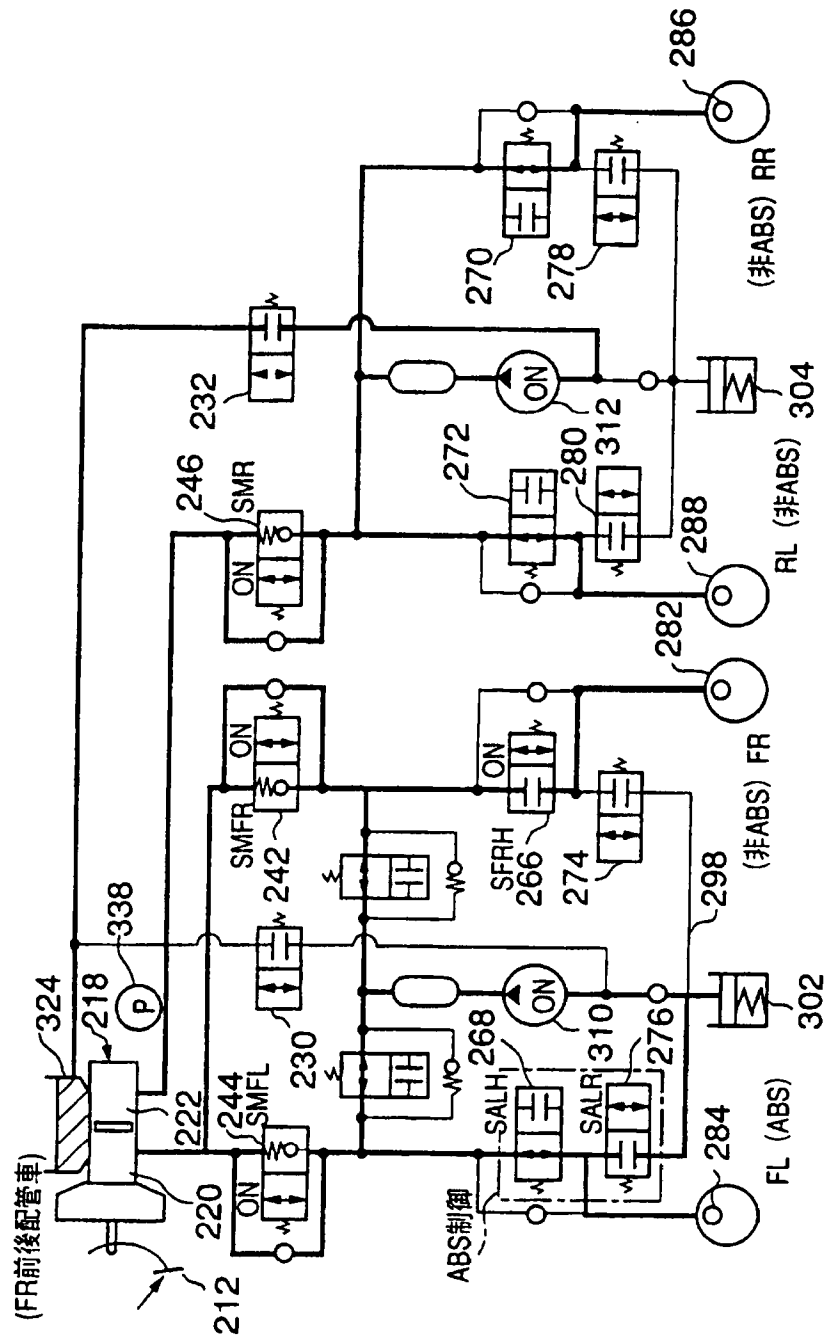
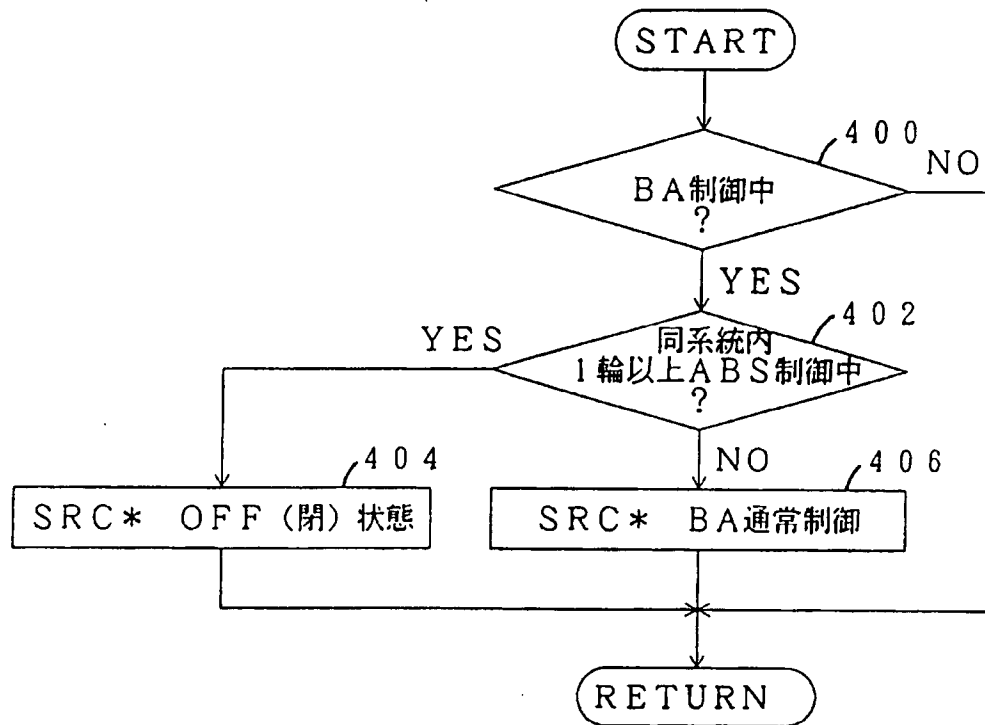


FIG. 18



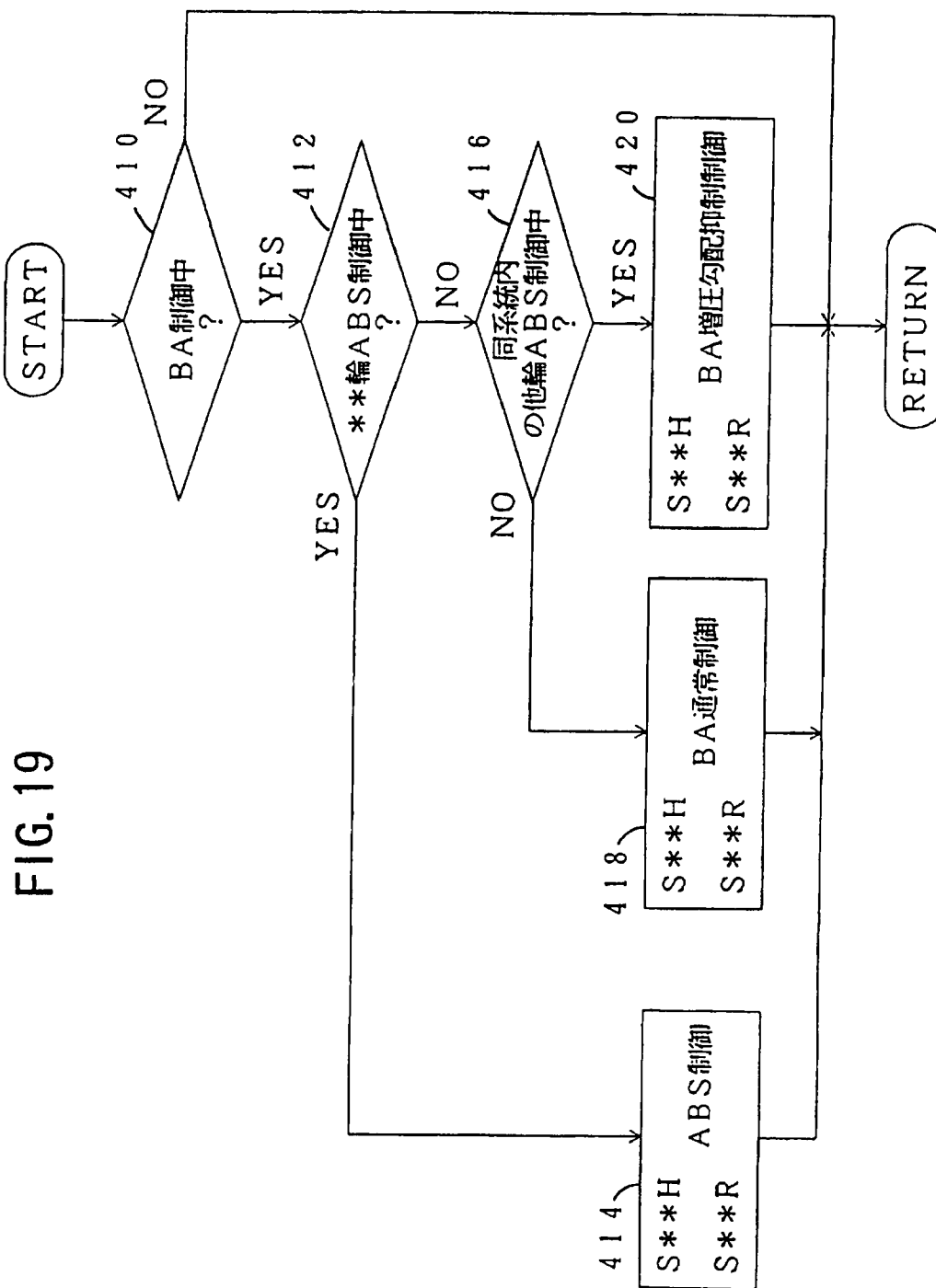


FIG. 20

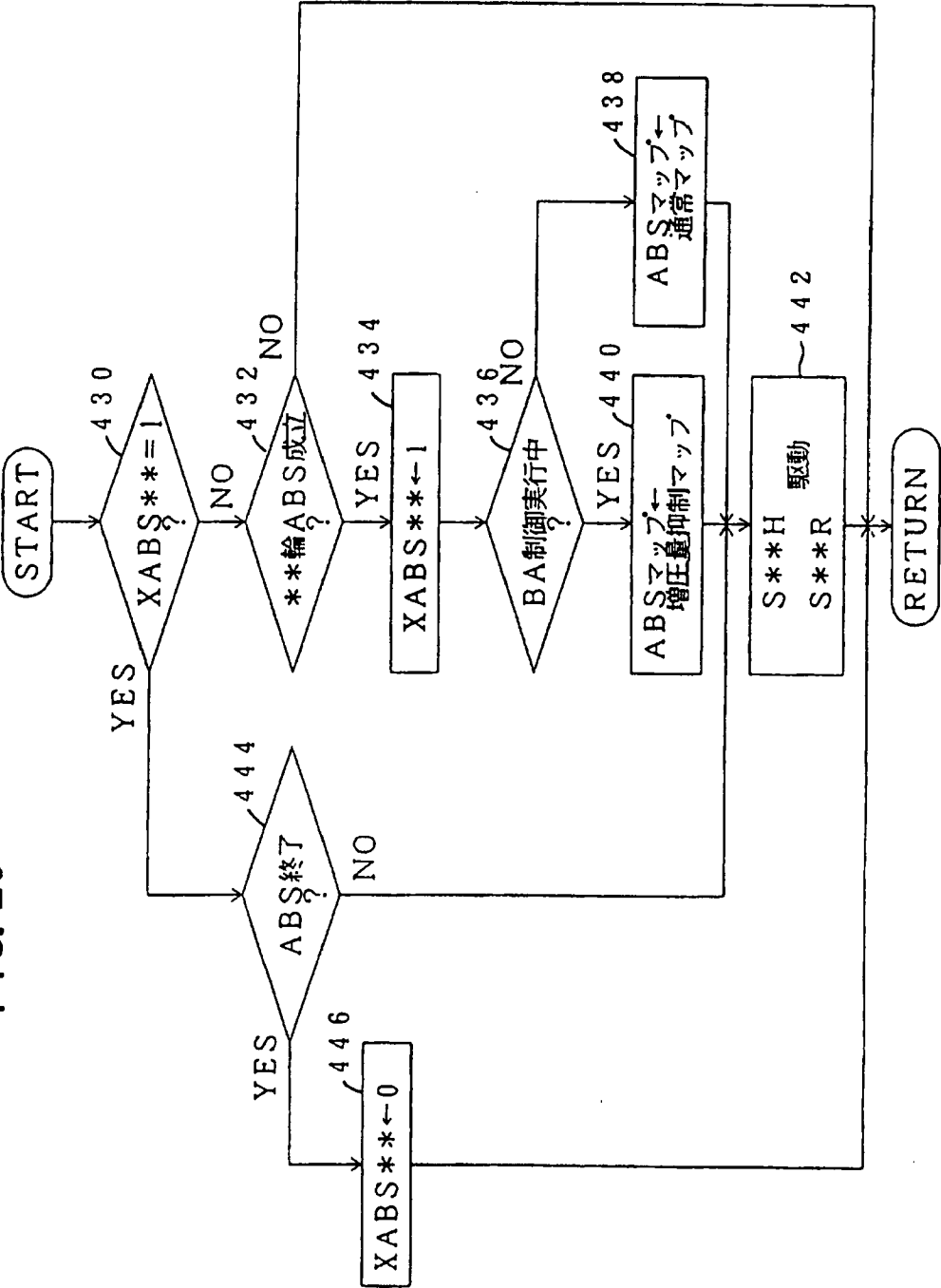


FIG. 21

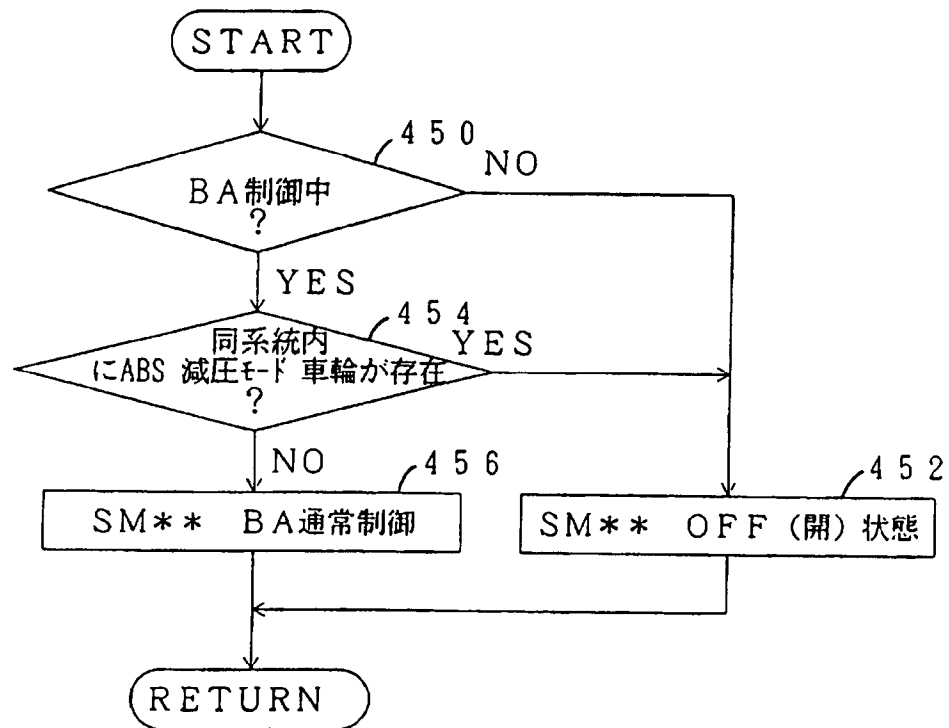


FIG. 23

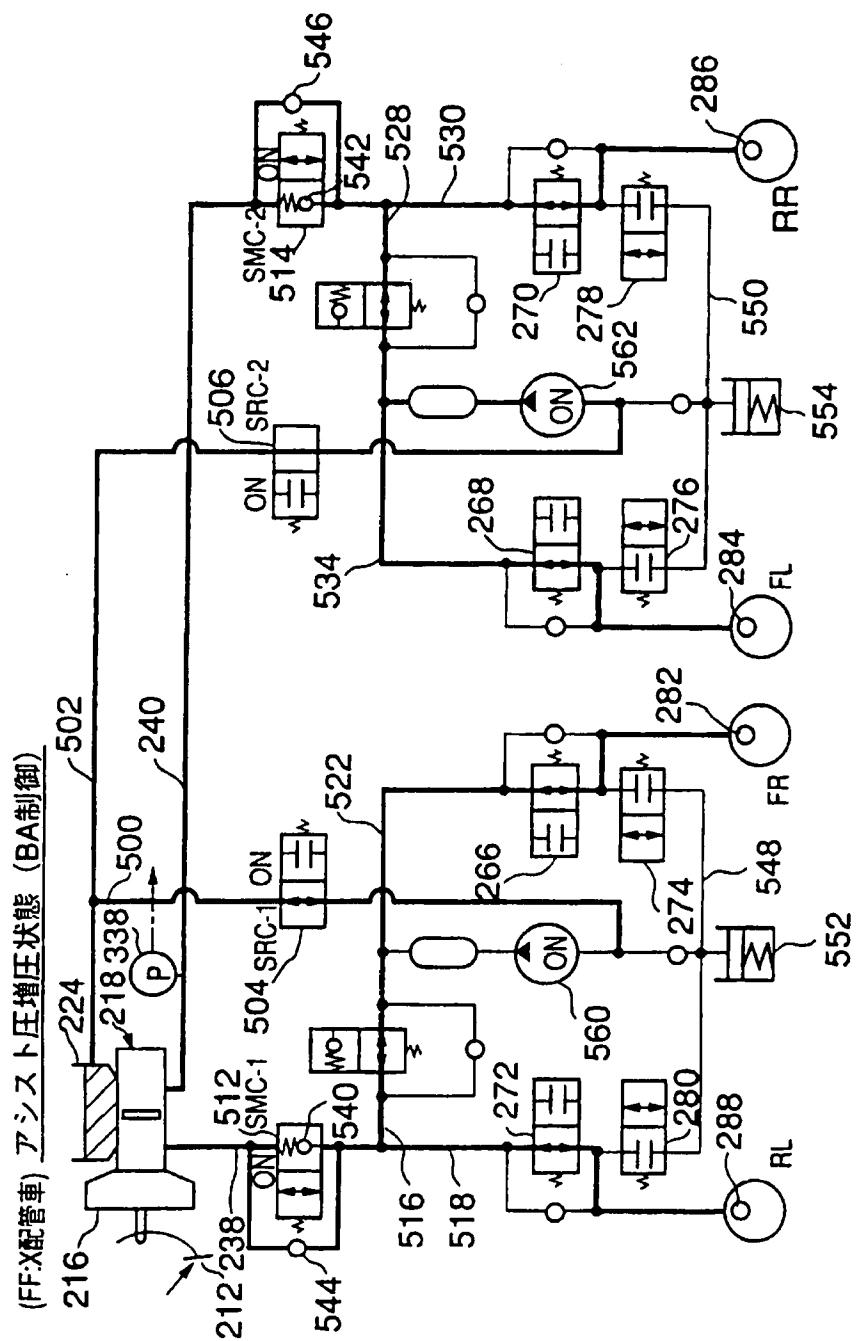


FIG. 24

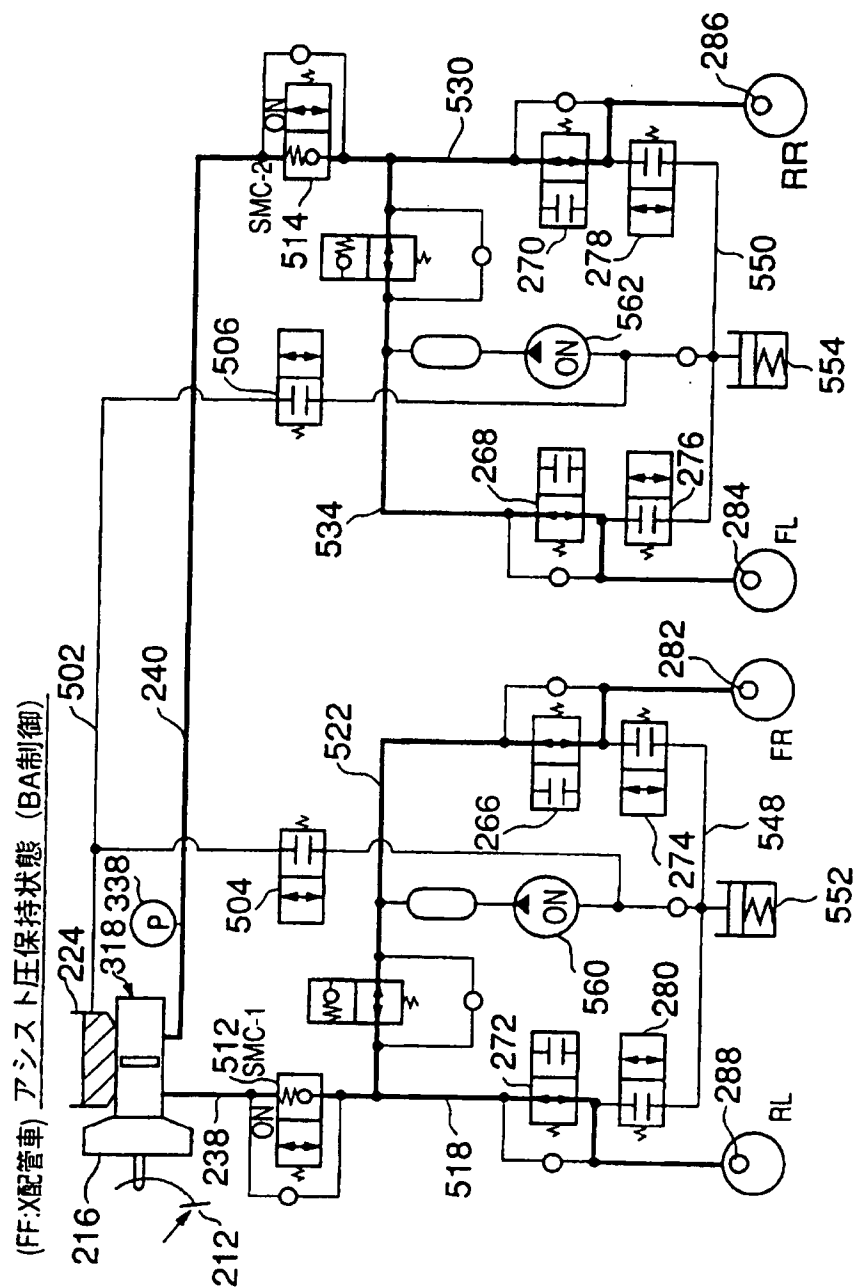


FIG. 25

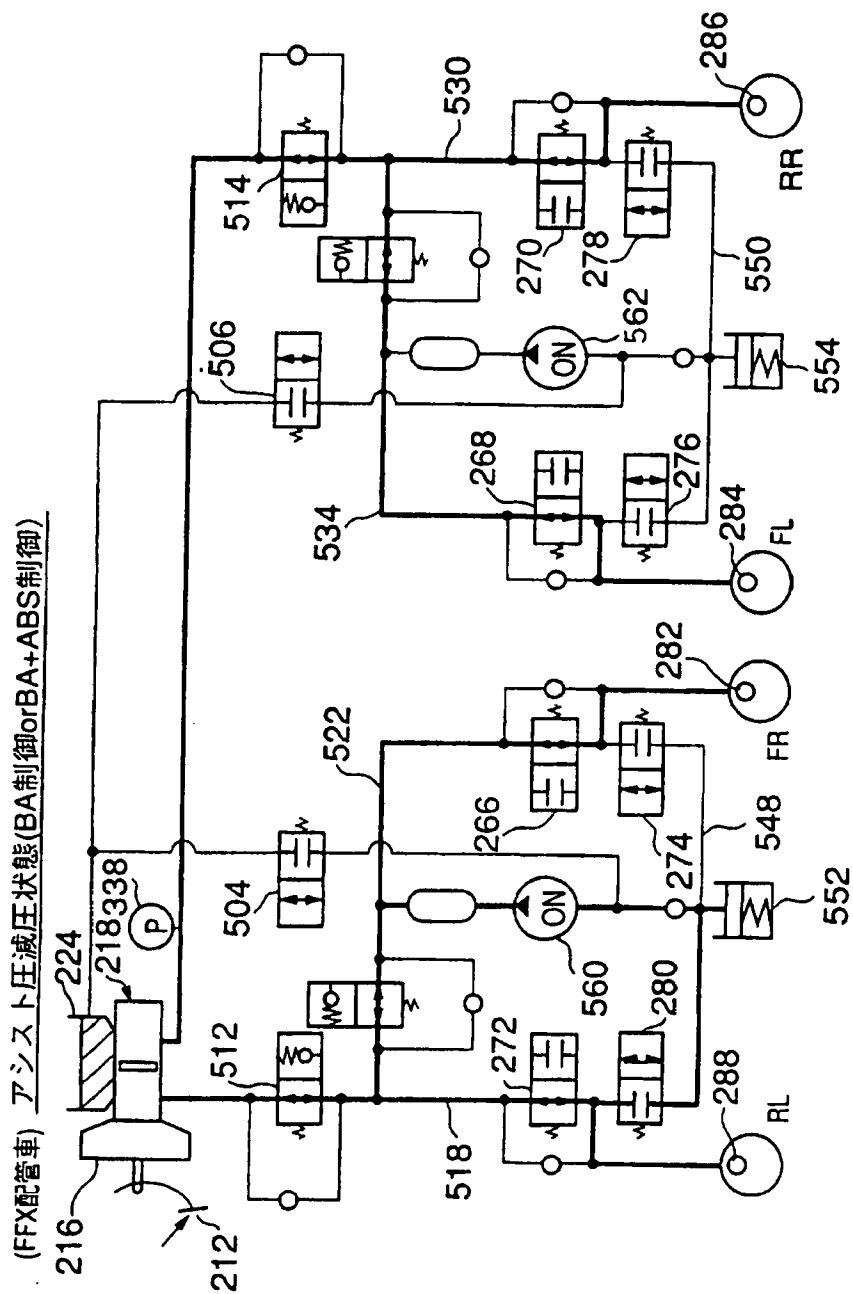


FIG. 26

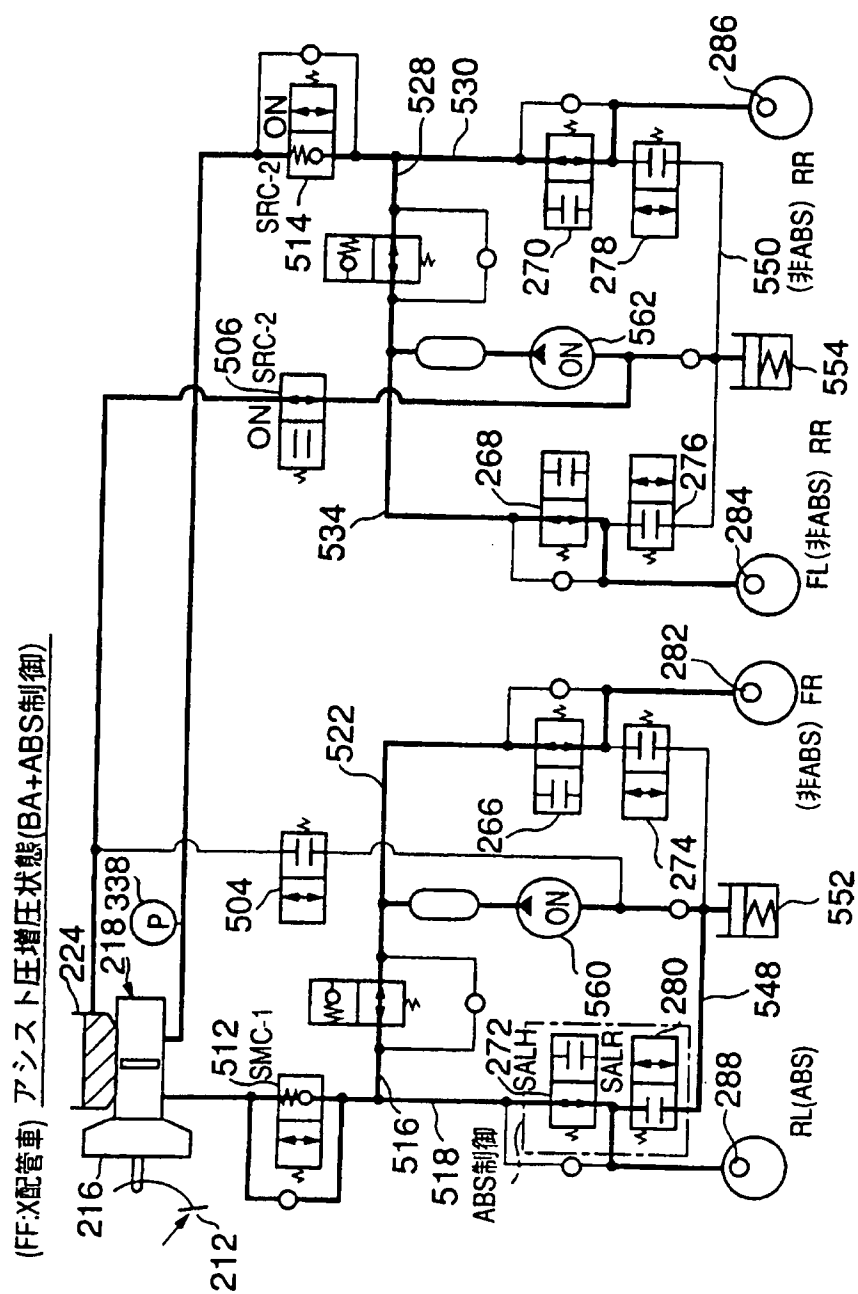
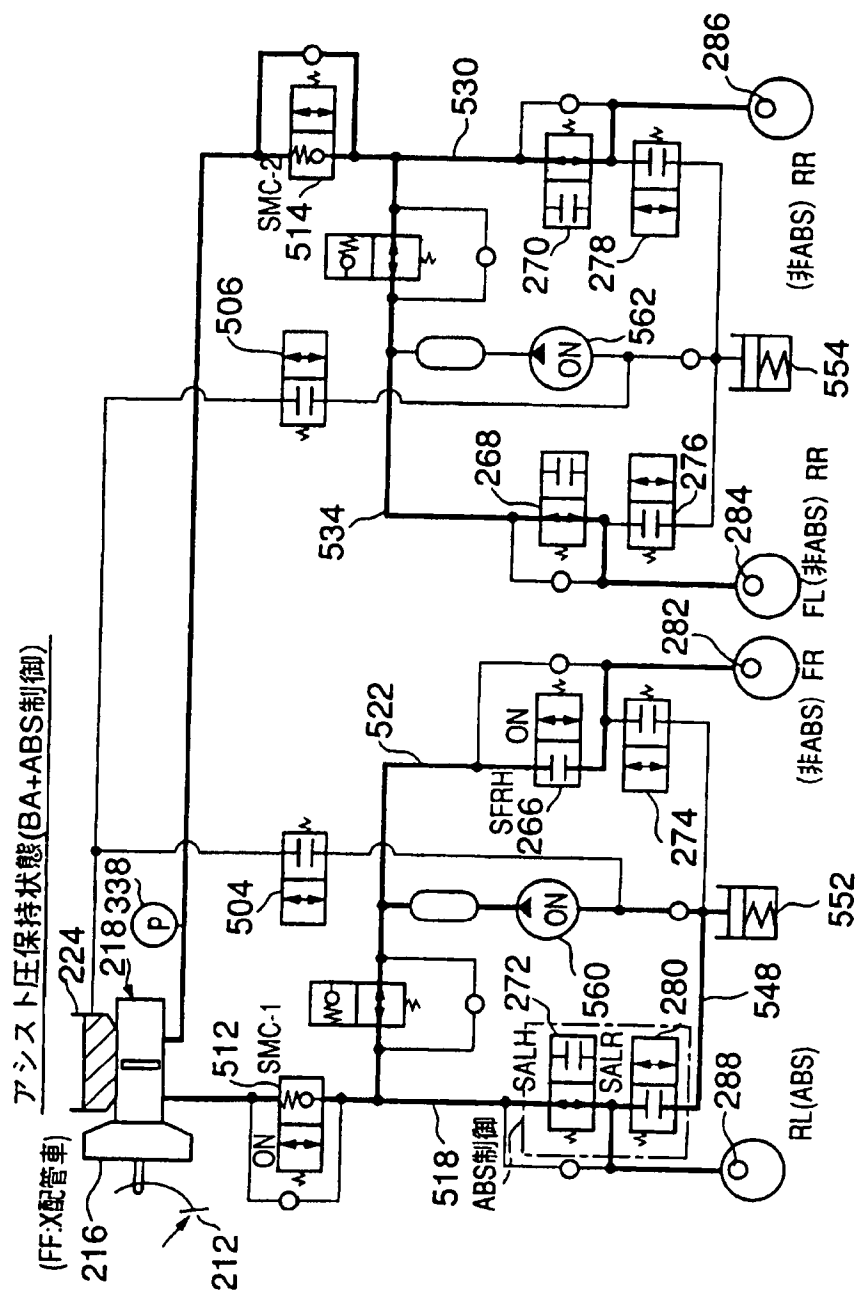


FIG. 27



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/02509

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl⁶ B60T8/00, B60T8/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl⁶ B60T8/00, B60T8/34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho	1926 - 1996	Jitsuyo Shinan Toroku
Kokai Jitsuyo Shinan Koho	1971 - 1997	Koho
Toroku Jitsuyo Shinan Koho	1994 - 1997	1996 - 1997

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 5-50908, A (Akebono Research and Development Center Ltd.), March 2, 1993 (02. 03. 93), Page 2, left column, lines 2 to 13 (Family: none)	1 - 8
A	JP, 7-315187, A (Fuji Heavy Industries Ltd.), December 5, 1995 (05. 12. 95), Page 2, left column, lines 2 to 15 (Family: none)	1 - 8
A	JP, 4-121260, A (Toyota Motor Corp.), April 22, 1992 (22. 04. 92), Page 4, lower part, right column, lines 5 to 18 (Family: none)	1 - 8
P	JP, 9-86372, A (Jidosha Kiki Co., Ltd.), March 31, 1997 (31. 03. 97), Page 2, right column, line 44 to page 3, left column, line 9 (Family: none)	1 - 8

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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Date of the actual completion of the international search

September 18, 1997 (18. 09. 97)

Date of mailing of the international search report

October 7, 1997 (07. 10. 97)

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A. 発明の属する分野の分類 (国際特許分類 (IPC))		
Int. Cl ⁸ B60T8/00, B60T8/34		
B. 調査を行った分野		
調査を行った最小限資料 (国際特許分類 (IPC))		
Int. Cl ⁸ B60T8/00, B60T8/34		
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日本国実用新案公報 1926-1996 日本国公開実用新案公報 1971-1997 日本国登録実用新案公報 1994-1997 日本国実用新案登録公報 1996-1997		
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A	J P, 5-50908, A (株式会社曙ブレーキ中央技術研究所) 2. 3月. 1993 (02. 03. 93) 第2頁左欄第2-13行 (ファミリーなし)	1-8
A	J P, 7-315187, A (富士重工業株式会社) 5. 12月. 1995 (05. 12. 95) 第2頁左欄第2-15行 (ファミリーなし)	1-8
A	J P, 4-121260, A (トヨタ自動車株式会社) 22. 4月. 1992 (22. 04. 92) 第4頁下段右欄第5-18行 (ファミリーなし)	1-8
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国際調査を完了した日 18. 09. 97		国際調査報告の発送日 07.10.97
国際調査機関の名称及びあて先 日本国特許庁 (ISA/J P) 郵便番号100 東京都千代田区霞が関三丁目4番3号		特許庁審査官 (権限のある職員) 藤井 新也 電話番号 03-3581-1101 内線 3328

C (続き) . 関連すると認められる文献		
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P	J P, 9-86372, A (自動車機器株式会社) 31. 3月. 1997 (31. 03. 97) 第2頁右欄第44-第3頁左欄第9行 (ファミリーなし)	1-8

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 9/127, 38/19, 39/39, 38/28	A1	(11) International Publication Number: WO 00/44350 (43) International Publication Date: 3 August 2000 (03.08.00)
(21) International Application Number: PCT/EP00/00598 (22) International Filing Date: 26 January 2000 (26.01.00) (30) Priority Data: 99101480.4 27 January 1999 (27.01.99) EP (71) Applicant (for all designated States except US): IDEA AG [DE/DE]; Frankfurter Ring 193a, D-80807 Munich (DE). (72) Inventors; and (75) Inventors/Applicants (for US only): CEVC, Gregor [DE/DE]; Erich-Kästner-Weg 16, D-85551 Kirchheim (DE). CHOPRA, Amla [IN/IN]; A/21A, Ashok Vihar, Ohase 1, Delhi 110 052 (IN). STIEBER, Juliane [DE/DE]; Clemensstrasse 74, D-80769 Munich (DE). (74) Agent: VOSSIUS & PARTNER; P.O. Box 86 07 67, D-81634 Munich (DE).		(81) Designated States: AU, BR, CA, CN, HU, JP, KR, MX, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: TRANSNASAL TRANSPORT/IMMUNISATION WITH HIGHLY ADAPTABLE CARRIERS (57) Abstract The invention deals with the transport of preferably large molecules across nasal mucosa by means of specially designed, highly adaptable carriers loaded with said molecules. One of the purposes of making such formulations is to achieve non-invasive systemic delivery of therapeutic polypeptides, proteins and other macromolecules; the other intent is to overcome circumstantially the blood-brain barrier by exploiting the nasal cavity to enter the body and then to get access to the brain. A third intent is to achieve successful protective or tolerogenic immunisation via nasal antigen or allergen administration.		

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Transnasal transport/immunisation with highly adaptable carriers

The invention deals with the transport of preferably large molecules across nasal mucosa by means of specially designed, highly adaptable carriers loaded with said molecules. One of the purposes of making such formulations is to achieve non-invasive systemic delivery of therapeutic polypeptides, proteins and other macromolecules; the other intent is to overcome circumstantially the blood-brain barrier by exploiting the nasal cavity to enter the body and then to get access to the brain. A third intent is to achieve successful protective or tolerogenic immunisation via nasal antigen or allergen administration.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art of the present invention. Further incorporated by reference is the complete disclosure content of the co-pending application filed in the name of IDEA AG and bearing the title "Noninvasive vaccination through skin".

Nasal delivery has been explored extensively over the last decades and was discussed repeatedly as an alternative to the systemic delivery of drugs, especially peptides and proteins, which normally must be injected. Nasal delivery also attracted interest owing to the fact that it avoids the hepatic first-pass effect, the problem of degradation in nasal cavity notwithstanding, which creates a pseudo-first-pass effect (Sarkar, 1992). The latter difficulty prompted chemical or recombinant structural peptide or protein modifications to improve the stability and minimise the enzymatic cleavage of macromolecules in the nose (Wearley, 1991).

Some earlier reviewers (Illum, 1991; Wearley, 1991) expected that transnasal peptide delivery, supported by absorption enhancers, will provide a convenient, efficient means for the administration of protein and peptide therapeutics. More recent surveyors took less optimistic stance, however (Harris, 1993). Rapid metabolism and nonlinear pharmacokinetics of nasally delivered peptides (Wearley, 1991) are partly responsible for this. The other reasons are the anatomical and temporal barriers presented by the

nasal mucosa (Sarkar, 1992), and especially the intolerable side effects of most, if not all, methods currently in use for nasal delivery. This holds also true for efforts to deliver compounds with the aim to generate a protective immune response transnasally, which would represent a more natural way of antigen presentation than encountered by conventional injection. The adverse side effects observed with transnasal immunisation experiments are mainly due to the presence of immunoadjuvants (such as Cholera toxin (CT) or its fragment B, heat labile protein from *E. coli*, keyhole limpet hemocyanin, or other substances with ADP-ribosylating activity, for example), and/or molecules with a permeation enhancing activity, in addition to the antigen in the formulation for nasal delivery. While the former may be toxic, the latter are irritating to the immunised subject. Selectivity of immune response, moreover, cannot be achieved with unspecific stimulatory agents. Moreover, there is substantial variability in the resulting immune response after nasal antigen administration, probably due to the difficulty of depositing the immunogen on the sites in the nasal cavity with the lowest transbarrier transport resistance.

The human nasal cavities with a total volume of 15 mL and a total surface area of 150 cm² - which amounts to more than 1 m² if one allows for the surface corrugations - are covered by mucus and a mucosa 2 mm to 4 mm thick. Most of the cavity surface is lined by a respiratory epithelium, comprised of columnar cells, goblet cells, and ciliary cuboidal cells. The resulting permeability barrier is related to that of the oral cavity, with which it communicates and which is covered by a keratinised barrier tissue. In either case, the cells in the barrier are tightly packed and often sealed with the specialised intercellular lipid arrangements. Moreover, in either case, the permeability barrier is lowered by the topical use of substances which compromise the quality and packing of such lipidic seals and/or which increase the probability for molecular partitioning into the barrier. Deviant from the situation encountered in the mouth, from the nose foreign substances are cleared into the nasopharynx by the cilia, with an average speed of 5 mm/min. An exception is the upper region of nasal cavity, which contains no cilia but is covered by a pseudo-stratified olfactory neuroepithelium. The nasal subepithelium contains a dense vascular network and the venous blood from the nose passes directly into the systemic circulation.

Nasal route of delivery has been relatively unsuccessful to date when used for high molecular weight substances. Use of permeation enhancers did not improve the

situation sufficiently, largely due to the fact that such substances are generally poorly tolerated and of limited usefulness. The pharmacodynamics resulting from nasal drug delivery is also highly variable. Major reasons for this are the inconsistency in the site of deposition or in the delivery details, as well as changes in the mucous secretion and mucociliary clearance; the latter are compounded especially by the presence of allergy, hay fever, and the common cold in treated subjects (Harris, 1993). Protein degradation in mucosa is important as well (Sarkar, 1992). Despite this, numerous studies were done with buserelin, vasopressin, cholecystokinin, calcitonin, growth hormone and related substances (e.g. GHRH), erythropoietin, G-CSF, interferon, insulin, gonadotropin hormone releasing hormones (GnRH), and vasopressin analogues, the results of which are reviewed briefly in the following.

Systemic delivery of large drugs through the nose

Hexarelin (GH analogue; MW \approx 800). The GH response to the intranasal hexarelin administration (about 18 μ g/kg) was not significantly higher than that induced by an injection of 1 μ g GHRH/kg (Ghigo et al., 1996). On the other hand, the former kind of treatment did not significantly modify IGF-I but increased IGFBP-3 levels. Both IGF-I and IGFBP-3 levels were slightly but significantly increased by oral treatment with the drug as well (Ghigo et al., 1996).

Intranasal treatment with octreotide nasal powder, a somatostatin analogue (up to 2 mg TID, corresponding to a mean GH value below 5 μ g/L during 8 daytime hours), was well tolerated, with only mild side effects and no significant changes in the nasal mucosa. An improvement of the clinical picture was registered in all patients after a few days of octreotide nasal powder administration. Positive correlation was found between GH and IGF-I, GH and IGFBP-3, IGF-I and IGFBP-3, insulin and IGFBP-3 and insulin and IGF-I during chronic (3-6 months) treatment (Invitti et al., 1996).

Cholecystokinin (MW \approx 1050). The carboxy terminal octapeptide of cholecystokinin (CCK-8) has similar functions as native cholecystokinin (CCK), but lacks receptor selectivity and metabolic stability. Mediation of satiety via the A-receptor subtype can be used for management of obesity. This was also shown after intranasal administration of Hpa(SO₃H)-Nle-Gly-Trp-Nle-MeAsp-Phe-NH₂, the result of moving

the N-methyl group from Phe to Asp, which inhibited feeding in beagle dogs (Pierson et al., 1997).

After intranasal (10 μ g) and intravenous (0.25 μ g and 2.5 μ g) administration of an octapeptide derivative of cholecystokinin, the substance CCK-8 was shown to affect the auditory event related potential (AERP) in 20 healthy subjects. The effect was stronger in women than in men (Pietrowsky et al., 1996). Plasma CCK-8 concentrations after intranasal administration of 10 μ g CCK-8 were comparable to those of 0.25 μ g CCK-8 given i.v., but were substantially lower than those elicited by 2.5 μ g CCK-8 (Pietrowsky et al., 1996).

Vasopressin (MW = 1054). Vasopressin DGAVP (2 mg) was administered intranasally and orally to healthy subjects for 1 week. Peak levels were always observed at 15 min. The mean absorption and elimination half-life (around 8 min and 35-38 min, respectively) were similar for the two tested routes of administration, but the latter only had 0.7 % relative bioavailability (Westenberg et al., 1994).

In a double-blind, crossover study, subjects received on three different occasions 20 IU of (arginine)vasopressin (AVP) intranasally (IN), or 1.5 IU of AVP and saline solution i.v. Evoked potentials (ERPs) were recorded during the subject's performance on a auditory attention task. Plasma concentrations of vasopressin during task performance were enhanced after AVP, with the increase after i.v. administration of AVP exceeding 2000-fold that after AVP i.v. Intranasal administration of AVP substantially increased the P3 component of the ERP in contrast to the injection (Pietrowsky et al., 1996).

Acute (2 mg) and chronic, 2 weeks treatment (1 mg/day) with nasal DGAVP revealed an improved short-term memory for abstract words in males but not in females, with no positive effect on learning concrete words. Chronic, but not acute, treatment with DGAVP reduced the reaction time for scanning of digits in a memory comparison task (Sternberg paradigm) in both sexes (Bruins et al., 1995). In a different human study, arginine-vasopressin (AVP: 3x10 IU) enhanced memory performance after nasal administration. The late positive complex (LPC) elicited by oddball stimuli was not affected whereas the structural encoding task revealed an effect of the drug. In both studies, AVP intake resulted in a marked change of the scalp distribution of the P3 component, which is a prominent part of the LPC. Vasopressin was thus concluded to

influence the central nervous processing of the emotional content of stimuli (Naumann et al., 1991).

Subchronic treatment with vasopressin (40 IU/day) was shown to enhance nocturnal slow-wave sleep in 2 elderly subjects (Perras et al., 1996). However, the intranasal administration of vasopressin (DDAVP: 30 or 60 micrograms) had no general effect on pain perception in humans, but some other effects were observed (Pohl et al., 1996).

Buserelin (MW = 1239). Treatment of 40 women with endometriosis and 10 women with uterine leiomyoma by using GnRH agonist buserelin (200 µg, 3x daily, 6 months, intranasally) reduced AFS mean pelvic score from 24 to 7 and the size of the fibroids decreased by 69 % (Biberoglu et al., 1991).

Calcitonin (MW = 3432). Ichikawa et al. (1994) concluded that nasal (5, 10, 20 and 40 U/rat) and subcutaneous (5, 10 and 20 U/kg) administration of *Salmon calcitonin* on alternate days for 3 weeks, starting a week after ovariectomy, prevented the osteopenic changes, the invasive method being approximately 2-times more effective.

In a double-blind trial, the effect of intranasal administration of *Salmon calcitonin* on biochemical parameters of bone turnover in 32 patients immobilised for a prolapsed intervertebral disk was investigated (van der Wiel et al., 1993). Calcitonin in a dose of two times 200 IU/day inhibited by 40 % the increase in the fasting 2 h urinary hydroxyproline/creatinine ratio (OHP_r/Cr) and lowered by 80 % the increase in calcium/creatinine ratio (Ca/Cr). The decrease in serum 1,25-dihydroxyvitamin D after 10 days of immobilization was significantly less in the calcitonin-treated group than in the placebo group (14 versus 29%, respectively; $P < 0.05$). However, intranasal calcitonin, which was well tolerated, did not influence the pain scores as measured with a visual analog scale (van der Wiel et al., 1993).

Growth hormone (GH) releasing factor/s (MW = 5040). The current mode of growth hormone replacement therapy is daily subcutaneous (s.c.) injections given in the evening. This schedule is unable to mimic the endogenous pulsatile pattern of GH secretion, which might be of importance for the induction of growth and other GH actions (Laursen et al., 1996).

To simulate endogenous production of growth hormone the protein was administered on three occasions intranasally in doses of 0.05, 0.10 and 0.20 IU/kg, using didecanoyl-L- α -phosphatidylcholine as an enhancer (Laursen et al., 1996). On the other two occasions the patients received an s.c. injection (0.10 IU/kg) and an i.v. injection (0.015 IU/kg) of GH, respectively. The nasal doses and the s.c. injection were given in random order in a crossover design. Intravenous administration produced a short-lived serum GH peak value of 128 μ g/L. Peak levels were around 14 μ g/L after s.c. injection (50% bioavailability) and between 3 μ g/L and 8 μ g/L, respectively, after the three nasal doses (bioavailability between 4% and 9%). Serum insulin-like growth factor I (IGF- I) levels increased significantly after s.c. administration only. However, the data revealed that a closer imitation of the physiological GH pulses was achieved via the nose. Despite this the authors of the study concluded that GH administration is of limited importance for the induction of a metabolic response to GH (Laursen et al., 1996).

GHRP-2 is one of the most potent members of the GHRP family, which exerts its biological activity after oral, intranasal and i.v. administration. For example, the children who had a robust response to the injected GH-releasing factors also received intranasal GHRP-2, with significant, but not quantitated, response over a dose range of 5-20 μ g/kg per dose (Pihoker et al., 1995).

Insulin (MW = 5808). The problem of low bioavailability of insulin solutions given through the nasal mucosa was improved by using absorption enhancers or bioadhesive microspheres (Gizurason & Bechgaard, 1991; Illum & Davis, 1992). Bioavailability greater than 10% was measured but to date no corresponding formulation has found its way into the late clinical trials. The chief reason for this appears to be the severe damage to nasal mucosa caused by the commonly used permeation enhancers.

For example, following the administration of powder formulations comprising insulin and the permeation enhancer sodium tauro-24,25-dihydrofusidate (STDHF), the hypoglycaemic response and the serum insulin levels in sheep increased with STDHF/insulin molar ratio in the range 0 to 16.8 (Lee et al., 1991). The reason for this is increased mucosal permeability as well as reduced insulin aggregate size. The bioavailability ranges from 2.9% to 37.8% for the powder, and was reported to be

15.7% and 37.4%, respectively for the drops or spray containing STDHF/insulin = 8.4/1 mixture, and roughly proportionally to the enhancer concentration (Lee et al., 1991). To achieve a high bioavailability major changes in nasal mucosa had to be tolerated, however.

In humans, the 200 U insulin/mL formulation containing a blend of enhancers (didecanoyl-phosphatidylcholine (2 w-%), glycerol (1.6 w-%), 0.4 w-% fractionated coconut oil) and 0.2 w-% cholesterol resulted in appr. 8 % bioavailability, the highest values having been measured for the high dose (2x3 sprays of 50 μ L each), which also was most irritant (Drejer et al., 1991).

Cyclodextrins dissociate insulin hexamers into smaller aggregates, in dependence on structure and concentration. Hexamer dissociation was therefore speculated to be the reason for higher nasal absorption of the polypeptide (Shao et al., 1992). The relative effectiveness of various cyclodextrins for this purpose was reported to decrease from dimethyl- β -cyclodextrin (DM- β -CD) > α -cyclodextrin (α -CD) > β -cyclodextrin (β -CD), hydroxypropyl- β -cyclodextrin (HP- β -CD) > γ -cyclodextrin (gamma-CD). A direct relationship between absorption promotion and nasal membrane protein and lipid release was invoked to explain such sequence (Shao et al., 1992).

It is less clear why cationic chitosan enhances the absorption of insulin across the nasal mucosa of rat and sheep in a concentration dependent fashion, with optimum concentrations higher than 0.2% and 0.5% in rats and sheep, respectively, but overall efficiency of this procedure is only around 10% (Illum et al., 1994). Using didecanoyl-L- α -phosphatidylcholine as an enhancer results in 4% to 9% of nasal insulin bioavailability (Laursen et al., 1996).

G-CSF (MW = 19600). The relative bioavailability of rhG-CSF administered nasally in the rat was approximately 2%, compared to an s.c. injection, as evaluated from the immunologically active rhG-CSF concentration in rat plasma and the area under the curve (AUC) at t=8 h. Leukocyte stimulation counts suggested 5-10% availability at t=48 h. Relative bioavailability and pharmacological availability were increased 23 times and 3 times, respectively, by polyoxyethylene 9-lauryl ether (Laureth-9), but no increase in availability occurred with sodium glycocholate (Machida et al., 1993).

Absorption of dissolved recombinant human granulocyte colony-stimulating factors (rhG-CSF at pH 4) through the nose of rabbits was investigated with dimethyl- β -cyclodextrin added or without such excipient, which acts as barrier permeation enhancer. The proteins were absorbed and the total leukocyte numbers in peripheral blood increased in either case, but excipients improved the absorption of rhG-CSF appreciably (Watanabe et al., 1993). A subsequent pharmacokinetic and pharmacodynamic study (Watanabe et al., 1995) revealed that protein is absorbed through the nasal cavity from a solution, especially in the presence of alpha-cyclodextrin (α -CyD), which can act as carrier in the membrane. Good correlation was found between the logarithm of the area under the serum G-CSF concentration-time curve (AUC) and the area under the increased total blood leukocyte count-time curve (Watanabe et al., 1995).

Interferon (MW = 23000). Treatment of experimental rhinovirus colds in 38 adults by intranasal administration of recombinant interferon beta serine (MW = 18500) had no effect on illness rate or severity, but did decrease the frequency of virus shedding by the factor of 2 (on day 4) to 3 (on day 6). The course of middle-ear dysfunction associated with experimental colds was also positively affected by the drug (Sperber et al., 1992).

Erythropoietin (MW = 30400). The pharmacological availability of rh-EPO after intranasal administration without enhancers was compared to that of intravenous injections. The pharmacological activity was enhanced in low pH and hypotonic mannitol solution, which both compromise the barrier quality. This resulted in relative bioavailability of nasally applied drug between 7% and 4%, when estimated by different reticulocyte counting methods. (Shimoda et al., 1995).

Labelled dextrane (MW = 4100, 9000, 17500), applied nasally at the dose of 6.5 mg, was seen to pass mucosa in the presence of glycocholate (3 mg) and found in the blood in concentration range between 6 ng/mL and 21 ng/mL, which corresponds to app. 0.05%, 0.02%, and 0.01% for the three molecular sizes, respectively (Maitani et al., 1989).

In summary, the combined teachings of the prior art demonstrated that the likelihood of large molecules to pass nasal mucosa decreases strongly with increasing molecular weight. To date, the size of molecules administered successfully through the nose is typically < 1300 Da, and always below 3500 Da. Significant transport is achieved only with supporting permeation promoters and is, in a certain concentration range at least, proportional to the enhancer concentration. Enhancer concentration in the percentile range can ensure up to 30% drug (or label) bioavailability but more often values below 10% and typically of a few percent are obtained. High transfer efficiency is accompanied with strong local tissue damage. This causes unpleasant acute side effects and may, first, abrogate the nasal permeability barrier and, upon repeated use, provoke extensive keratinisation of the epithelium that finally reduces transnasal transport efficiency.

The success of transnasal transport is believed to rely on the loosening of ciliated-goblet, goblet-goblet, or ciliated-ciliated cell contacts, which also opens passages for the motion of water (McMartin et al., 1987). Procedures or substances which support the process either osmotically (as in the case of polysaccharide addition), physico/chemically (as in the case of surfactant addition) or biologically (as in the case of molecules which affect the cell biochemistry, including many drugs, cell adhesion or trans- and epicellular transport), can therefore improve drug delivery across the nasal mucosa. Translocation through the cells is possible, but probably rare, except, maybe, in the cases of some viral infections or applications. Materials, such as polymers of polyelectrolytes, which prolong the retention time of and increase the proximity between the molecules to be transported and cellular membranes, are useful for the purpose as well. The limit to this latter effect is set by ciliary motion, which tends to clear mucosal surface approximately every 30 min and transports the superficial material into the throat, and thus towards the gastrointestinal tract. Transport mediated by certain particles was contended to rely on this effect.

Particle delivery through the nose

Inhaled fine particles (Kanto loam dust, fly ash, carbon black, diesel exhaust particles (DEP), and aluminium hydroxide (alum)) appear to act as adjuvants, and accelerate the production of IgE antibody against pollen in female BDF1 mice; however, the

nature of the particles, their capacity to adsorb antigens, and/or their size seem to play only minor role in the process (Maejima et al., 1997).

Hollow spheres, according to Ting et al. (1992), are unsuitable for nasal delivery, owing to their rapid clearance and variable deposition pattern. Polyvinyl alcohol microparticles in the form of collapsed, solid spheres with the desired size for nasal deposition (10-200 μm) were therefore produced by spray-drying and spray-desolvation (Ting et al., 1992).

The above observation notwithstanding, several kinds of particulate suspensions were used in the nose, typically to elicit antibodies against the particle-associated antigens.

This includes so-called proteosomes comprising gp160 (Lowell et al., 1997) or influenza virus proteins. Another example are particles made from polymerised carbohydrates coated with a lipid (bi)layer.

It is important to realise, however, that in any nasal uptake study one should consider and allow for secondary redistribution. For example, the biodistribution of radioactivity from the purified major *Parietaria judaica* allergen after sublingual, oral, and intranasal administration in healthy human volunteers is similar. This is indicative of test material swallowing and absorption in the gastrointestinal tract (Bagnasco et al., 1997). In the intranasal case, transport to the pharynx by mucociliary clearance plays an important role as well, but a relevant fraction of the tracer is retained on the nasal mucosa for up to 48 hours after administration (Bagnasco et al., 1997).

Oral spill-over and the danger of false positive results

Proteins are absorbed in the gastrointestinal tract, albeit in small quantities. For example, ovalbumin (OVA) is absorbed in the stomach as well as from the GI tract into the blood and lymph circulation at levels of 0.007-0.008% and 0.0007-0.002% of applied dose; a higher dose in the latter case leads to relatively higher absorption (Tsume et al., 1996). Stomach absorption supplies nearly exclusively the blood, suggesting different mechanisms and/or routes of absorption between the stomach and the small intestine. OVA association with liposomes can improve the uptake about 2 to 3-fold, possibly owing to slower enzymatic degradation of OVA.

Often, the result of nasal and oral immunisation are very similar, suggesting that part of the effect of the former may be due to the spill over of the antigen into gastro-intestinal tract. Data obtained with human adenovirus type 5, used as a vector for heterologous DNA sequences, illustrate this (Flanagan et al., 1997).

Transnasal delivery into the central nervous tissue (CNS)

The access of substances to the brain is of paramount importance for the treatment of psychiatric and neurologic diseases. Transnasal route of delivery into the CNS was therefore tested for a few selected bioactive molecules.

To date, drug delivery into the CNS tissue by nasal administration has received little attention (Pescechnik & Price, 1996). Wheat-germ agglutinin coupled to horseradish peroxidase was demonstrated to be taken up by the cells of olfactory nerve, resulting in concentration in the olfactory bulb around 0.1% of applied concentration; the underlying principle is probably receptor-mediated endocytosis of WGA and subsequent trans-synaptic, retrograde transfer towards the brain. A similar mechanism is also possible in the case of viral infections in the nose.

For example, an intranasal instillation of vesicular stomatitis virus (VSV), a negative-sense RNA virus, may result in a lethal infection of murine and rat brain (Huneycutt et al, 1994). Within 12 h following intranasal inoculation of VSV, this antigen can be detected in the olfactory nerve layer of the ipsilateral olfactory bulb. Within 3-4 days post-inoculation (p.i.), VSV had disseminated into the glomeruli of the olfactory bulb as well as the anterior olfactory nuclei, ipsilateral to the VSV instillation. Within the glomeruli, VSV antigen is more prevalent in the granule cells than in the mitral cells. Correspondingly, the lateral olfactory tract, where axons of mitral cells course, remain VSV negative throughout 7 days p.i. By 7 days p.i., viral proteins are detected in several additional regions extending to the brainstem. The pattern of VSV immunoreactivity supports the picture of initial infection of the olfactory bulb glomeruli, with subsequent spreads via both ventricular surfaces and retrograde transport within axons of neuromodulatory transmitter systems enervating the olfactory bulb (Huneycutt et al, 1994).

Draghia et al. (1995) have demonstrated that it is possible to transfer the *Escherichia coli* lacZ gene in vivo into the central nervous system structures of rats after nasal instillation of replication-defective adenoviral vector AdRSV beta gal. Mitral cells from the olfactory bulb, neurons from the anterior olfactory nucleus, locus coeruleus and area postrema expressed beta-galactosidase for at least 12 days (Draghia et al., 1995). Parainfluenza type 1 vaccine virus also directly accesses the central nervous system by infecting olfactory neurons (Mori et al., 1996).

However, it would be highly desirable to have a convenient and reliable transnasal transport system for the compounds that are capable of and intended to generate a protective immune response without simultaneously generating a variety of adverse side effects. Common types of non-invasive applications, including oral immunisation, often do not elicit the desired immune response. Many injectible vaccines also do not provide optimum antibody isotype pattern, mainly due to the unnatural route of antigen entry into the body. Transnasal immunisation remains problematic owing to the large size of typical immunogen which is subject to similar restrictions as the transport of pharmaceutically active compounds across the nasal mucosa.

In conclusion, although the prior art has tested various approaches to transnasal delivery it has hitherto failed to provide a convincing principle for convenient and well tolerated transfer of compounds, such as pharmaceutically active substances, immunogens/antigens or allergens, through the nasal barrier, in particular if said compounds are large. The solution to said technical problem, i.e. the provision of a suitable system, is provided by the embodiments characterised in the claims.

Accordingly, the present invention relates to use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the

penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, such droplets then acting as carriers, for the transnasal administration of pharmaceutically active compounds, antigens, allergens, mixture of antigens and/or mixture of allergens.

These compounds, antigens or allergens do not cross the nasal mucosa in a practically meaningful quantity on their own without causing unacceptable side effects.

As regards the above recited values of up to 99%, it is to be noted that values below 50% of the former relative concentration are particularly useful, with values below 40 rel-% or even around and below 30 rel-% being even more advantageous, whereas in the case of droplets which cannot be solubilised by the more soluble component relative concentrations which exceed the above mentioned relative concentrations by the factor of up to 2 are most preferred.

Formulations including the above-referenced penetrants are described in detail in DE 41 07 152, PCT/EP91/01596, PCT/EP96/04526, and DE 44 47 287, which are incorporated herewith by reference. Relevant information useful for penetrant manufacturing and loading with various macromolecular actives, which are too big to permeate through the barrier, is given in patent application PCT/EP98/06750, also incorporated herewith by reference.

More general information on lipid suspensions can be found in the handbook dealing with 'Liposomes' (Gregoriadis, G., ed., CRC Press, Boca Raton, FL, Vols 1-3, 1987), in the book 'Liposomes as drug carriers' (Gregoriadis, G., ed., John Wiley & Sons, New York, 1988), or in the laboratory manual 'Liposomes. A Practical Approach' (New, R., Oxford-Press, 1989). The properties of phospholipids, which can be used conveniently to prepare bio-compatible immunopenetrants, are reviewed in 'Phospholipids Handbook' (Cevc, G., ed., Dekker, New York, 1995).

The manufacturing temperature for said penetrants is normally chosen in the 0°C to 95°C range. Preferably, one works in the temperature range 10-70°C, most frequently at temperatures between 15°C and 45°C, under all circumstances below the temperature at which any important formulation ingredient would undergo an irreversible change in the composition or physical state. These temperatures can be determined by the person skilled in the art using his common general knowledge and the teachings of the various documents cited in this specification. (For reference: the skin temperature is normally around 32°C.) Other temperature ranges are possible, most notably for the systems containing freezable or non-volatile components, cryo- or heat-stabilised formulations, etc.

If required to maintain the integrity and the desired properties of individual system components, carrier formulations can be stored in the cold (e.g. at 4°C), with or without associated active agents. It is also possible, and sometimes sensible, to manufacture and store the preparation under an inert atmosphere, e.g. under nitrogen. The shelf-life of carrier formulation, moreover, can be extended by using substances with a small number of double bonds, that is, by a low degree of unsaturation, by choosing peroxide-arm ingredients, by including antioxidants, chelators, and other stabilising agents, or by preparing the agent loaded penetrants *ad hoc* or *in situ*, e.g. from a freeze dried or dry mixture.

The term "two forms of a substance" in connection with this invention means two ionization states or salt forms of the same substance, two different complexes of such substance, etc.

"Non-invasive administration" or "non-invasive delivery" in this specification denotes application on or transport through the nasal mucosa.

"Nasal administration", in the context of this document, refers to applications of test material, whether by direct intranasal intubation, spontaneous sniff of a drop of the test fluid, or an inhalation of the sprayed test-fluid into the nose, independent of precise site of impact or deposition.

The term "penetration" in this application describes non-diffusive motion of large entities across a barrier. This process is believed to involve penetrant adaptation to the

otherwise confining pores in the barrier, perhaps in association with a transient, selective, and reversible decrease in the barrier resistance.

The term "permeation" refers to a diffusion across the semipermeable barrier and is typically driven by the permeant concentration gradient across the barrier.

A penetrant, consequently, is an entity comprising a single molecule or an arrangement of molecules too big to permeate through a barrier but capable to cross the barrier owing to the penetrants adaptability to the shape and/or diameter of the otherwise confining passages (pores) of a barrier. This adaptability is seen from the fact, for example, that penetrants more than twice bigger than the pore diameter will cross the bilayer without being fragmented down to the pore size. A permeant, on the other hand, is an entity that can permeate through the semi-permeable barrier, such as the skin. A penetrant in an external field experiences a driving force proportional to the nominal penetrant size and to the applied field, which may occur naturally. Such a force, which on the intact, non-occluded skin is believed to originate from the water concentration gradient across the stratum corneum, can result in a penetrant motion through the barrier, including the skin, if the force is strong enough either to deform the penetrant or else to widen the passages in the barrier sufficiently to elude the problem of size exclusion, or both.

A permeant, on the other hand, is a molecule diffusing, or at least capable of diffusion, across the semi-permeable barrier.

The above-referenced penetrant is typically an ultra-adaptable entity comprising several components. Said penetrant, in the widest sense of the word, is a supra-macromolecular body that can pass spontaneously through the permeability barrier with pores much smaller than the penetrant diameter, and thus transport material from the application to the destination site on either side of the barrier. In order to meet this goal, the penetrant must adjust its properties, most notably its deformability, to the shape and size of the pores in a barrier. This typically occurs under the influence of a strong driving force or a pressure acting on all molecules in the penetrant. Gradients which do not depend on the penetrant concentration, such as hydration or external electric potential difference across the barrier, were shown to serve this purpose.

Lipid aggregates in (quasi)metastable state, and of the nature described above in connection with the invention, most often behave as highly adaptable penetrants, especially when they have the form of a tiny droplet surrounded by one or a few membranes (bilayers) (Cevc et al., 1997; Cevc et al., 1998). Owing to membrane metastability, unusually high local bilayer curvature can develop at the sites of transient, local membrane destabilisation without compromising the overall aggregate integrity. From the composition point of view, such ultra-adaptable and self-regulating vesicles typically consist of a suitably chosen lipid mixture. In order to change conventional lipid vesicles, liposomes, into the optimised vesicles (Transfersomes) one can add, for example, suitable edge-activators into the aggregate membrane (Cevc et al., 1998). Alternatively, molecules which change the system deformability after complexation with or binding to the basic aggregate ingredient can be used. Often, but not necessarily, the activators belong to the class of surfactants below the saturation or solubilization concentration, which in the latter case gives rise to mixed micelles formation. This is important as solubilised lipids, in the form of mixed lipid micelles, can cross the pores sufficiently wider than the micelle diameter but are incapable of enforcing channel opening in the biological tissues, which can be widened and trespassed by the mixed lipid vesicles, however. The postulated reason for this – to which the applicant does not wish to be bound – is the much greater aggregation number of the latter kind of aggregate which translates into the greater sensitivity to external, transport-driving gradients, such as the water activity gradient, and which is then capable of paying the energetic price for the pore or channel opening in the barrier.

The present invention is, in view of the prior art, particularly surprising since ultradeformable lipid vesicles would seem unsuitable for the purpose of transnasal delivery taken that they were reported to date to cross barriers, such as skin, only under non-occlusive conditions, that is, in the presence of a strong trans-barrier water concentration gradient (Cevc et al. 1995; Paul and Cevc, 1995), which is believed not to exist in the strongly hydrated nasal mucosa.

It was unexpectedly found that macromolecules in association with highly adaptable penetrants, typically in the form of mixed lipid vesicles, are transported across nasal mucosa despite the high water content in this mucosa and in the exhaled air saturated with humidity. Concluding from the fact that several successfully tested formulations of

such carriers caused no irritation in the nose it is inferred that the aforementioned transport does not rely on damaging the barrier, such damage being the reason for more conventional transport of macromolecules from a solution across the nasal mucosa. Rather than this, it is reasoned (wherein the applicant does not wish to be bound by theory) that said transport relies on the carrier penetration through the barrier, which should not occur in a very humid surrounding.

It is furthermore taught in accordance with the invention that increasing the concentration of the surface active molecules, which can act as permeation enhancers, decreases the efficiency of corresponding protein transport across the nasal mucosa, at least when the solubilisation point of the carriers has been reached. This finding is unexpected in view of the fact that the art teaches that the bioavailability of nasally administered macromolecules typically gets higher with increasing permeation enhancer concentration.

A third unexpected finding is that carrier-mediated delivery of macromolecules across the nasal mucosa can mediate a relatively efficient transport of large molecules into the central nervous system (CNS). The influx is seen relatively soon after the drug administration into the nasal cavity when the large molecules are associated with the carriers. This could be due to the transport of carrier-associated drugs across the nasal mucosa and subsequent uptake of drug-laden carriers into the olfactory nerve, through which the drug could be carried towards and into the CNS by the retrograde transport; such transport has already been postulated and was tested with individual molecules (Pasechnik-V; Price-J. Exp. Opin. Invest. Drugs; 5: 1255-1276); the approach was not used, to the best of the applicant's knowledge, in combination with particulates to date. An alternative explanation would involve the carrier-mediated macromolecular delivery into the peri-nasal lymphatic system, which has been reported to communicate with the central nervous system (Kida-S; Pantazis-A; Weller-RO. Neuropathol. Appl. Neurobiol. 1993; 19: 480-448).

A fourth surprising result achieved in accordance with the present invention is that the referenced penetrants allow a successful and preferably protective transnasal immunisation with large immunogens. The use of highly adaptable antigen- or immunogen-carriers for the purposes of immunotherapy is expected to or has been shown in accordance with the present invention to provide all the benefits of more

conventional nasal vaccinations in addition to the safety and robustness of administration. Improved safety would reflect the choice of the non-toxic and non-irritating carrier ingredients. Better reproducibility could result from the greater ability of the specially designed carriers, compared to that of the antigens or immunoadjuvants used alone to overcome the nose barrier. Taken the expectation that different carrier populations loaded with the individual antigens could be combined into a final multi-valent vaccine formulation the capability of invented technology to meet the trend in immunotherapy is given.

It stands to reason that non-toxic and „gentle“ formulations containing merely bio-compatible or natural, body-like ingredients, which protect the body faster and/or better than the corresponding antigen injections, would be preferred to the latter and would have a substantial commercial value.

In accordance with the present invention it is recommended to choose the penetrant characteristics, especially the deformability, concentration, or composition of the mixed lipid aggregates, so as to control the rate or the efficiency of penetrant-mediated transport.

In the process of optimisation of the formulation and/or administration it may be convenient to determine the flux of drug or agent loaded penetrants through the pores in a well-defined barrier as a function of suitable driving force or pressure, which act across the barrier, and then to describe the data by a convenient characteristic curve which, in turn, is employed to optimise the formulation or application further.

The pharmaceutically acceptable form of the agent may be given in a variety of final formulations, optionally, and depending on the purpose of the administration, in combination with diverse secondary agents. Such agents will be explained in more detail later in the text and may be, for example, bacterial compounds or other immunomodulations.

Furthermore, the present invention relates to the use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a

substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains as a carrier for the preparation of a pharmaceutical, preferably a vaccine composition for transnasal administration. It is preferred that these molecules used on their own do not cross the nasal mucosa in practically useful quantity without causing unacceptable side effects.

The carrier is combined with the pharmaceutically active ingredient prior to the administration, e.g. when formulating said pharmaceutical composition. As regards the further explanations, description of advantages etc., of this and the following embodiments, reference is made to the respective description in connection with the first embodiment described herein above. It is further to be understood in accordance with the present invention that more than one type of antigen, allergen or pharmaceutically active ingredient or combinations thereof may be formulated into said pharmaceutical composition.

Additionally, the present invention relates to the use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the

substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains in combination with a pharmaceutically active ingredient or an allergen or an antigen for the preparation of a transnasally administerable pharmaceutical composition for the treatment of infective diseases, endocrine disorders, preferably hypopituitarism, diabetes, hyperthyroidism, thyroiditis, most preferably Hashimoto's thyroiditis, subacute thyroiditis; adrenal disorders, preferably Addison's disease, secondary adrenal insufficiency, Cushing's syndrome; gastrointestinal disorders, preferably Crohn's disease, colitis; hemorrhagic diseases, preferably hemophilia, leukopenia, hypereosinophilic syndrome; musculoskeletal and connective tissue disorders, preferably rheumatoid arthritis, Sjögren's syndrome, Bechet's syndrome, lupus, scleroderma, polymyositis/dermatomyositis, polymyalgia rheumatica and temporal arthritis, polyarteriosis nodosa, Wegener's granulomatosis, mixed connective tissue disorder, ankylosing spondylitis, psoriatic arthritis, osteoarthritis, Paget's disease, sciatica, bursitis, tendonitis or tenosynovitis, epicondylitis, fibromyalgia, eosinophilic fasciitis; neurological disorders, preferably pain, singultus, vertigo, seizure disorders, sleep disorders, transient ischemic attacks, spinal cord injury, demyelinating diseases, nerve root disorders, myasthenia gravis; psychiatric disorders, preferably drug dependence, neuroses, mood disorders, schizophrenic disorders, delusional disorders; for oncological purposes and/or for treatment in the field of gynecology, preferably for the treatment of dysmenorrhea, menopause, chronic anovulation, premature ovarian failure, endometriosis, infertility; and/or for treatment in the field of immunology, preferably transplant rejection, hyposensitisation, allergen immunotherapy or prophylactic vaccination.

The term „allergen“ is used in this invention to describe materials of endogenous or xenogenic, e.g. animal or plant, origin which result in an undesired immune response of the body exposed to such an allergen, often resulting in an acute hypersensitivity reaction. Allergising microbes or parts thereof (e.g. of mite), parts of plants (e.g. pollen) or animal (e.g. hair and skin debris), but also man made and inorganic

substances belong to this group. On the other hand, nearly any part of the human body, if incorrectly processed by or exposed to the body's immune system, can result in an auto-immune response and lead to the allergic reaction to such a substance. In the narrower interpretation, used when so stated, an allergen is a substance, a group, or an arrangement of substances causing immediate hypersensitivity reactions in the body that could be diminished, or even eliminated, by an immunotherapy, whether done non-invasively through the nasal mucosa or not.

An „antigen“ is a part of a pathogen or an allergen in its natural form or after fragmentation or derivatisation. More generally, the word antigen denotes a macromolecule or a fragment thereof, any haptenic moiety (for example, a simple carbohydrate, complex carbohydrate, polysaccharide, deoxyribonucleic acid), in short, any molecule recognized by a body's antibody repertoire and possibly capable of antibody induction when administered in the system. A macromolecular antigen is defined as an antigen that is known to or believed to cross spontaneously the nasal barrier only in quantity too small for the desired practical purpose. Thus, macromolecules are molecules that, on their own, do not cross the nasal mucosa in practically useful quantity without causing unacceptable side effects.

The term "a mixture of antigens" or "a mixture of allergens" means, in accordance with the present invention, the combination of at least two antigens and/or allergens. It is envisaged that also mixtures of antigens and allergens can be used according to the present invention.

Furthermore, the present invention relates to a pharmaceutical composition for transnasal administration comprising a carrier which is a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the

penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains and a pharmaceutically active ingredient.

In a preferred embodiment of the use or the pharmaceutical composition of the present invention the pharmaceutically active ingredient is an adrenocorticostaticum, an adrenolyticum, an androgen or antiandrogen, an antiparasiticum, an anabolicum, an anaestheticum or analgesicum, an analepticum, an antiallergicum, antiarrhythmicum, antiarteroscleroticum, antiasthmaticum and/or bronchospasmolyticum, an antibioticum, an anti-infective agent, an antidepressivum and/or antipsychoticum, an antidiabeticum, an antidot, an antiemeticum, antiepilepticum, antifibrinolyticum, anticonvulsivum or anticholinergicum, an enzyme, a coenzyme or the corresponding enzyme inhibitor, an antihistaminicum (and combinations thereof) or antihypertonicum, an antihypotonicum, an anticoagulant, antimycoticum, antimyasthenicum, an agent against Morbus Alzheimer or Morbus Parkinson, an agent for ACS therapy, an antiphlogisticum, antipyreticum, antirheumaticum, antisepticum, a respiratory analepticum or a respiratory stimulant, a broncholyticum, cardiotonicum, chemotherapeuticum, a coronary dilatator, a cytostaticum, a diureticum, a ganglium-blocker, a glucocorticoid, an anti-flew agent, a haemostaticum, hypnoticum, an immunoglobuline or its fragment or any other immunologically active substance, such as an immunomodulator, a bioactive carbohydrate (derivative), a contraceptive, an anti-migraine agent, a corticosteroid, a muscle relaxant, a narcoticum, a neurotherapeuticum, a (poly)nucleotide, a neurolepticum, a neurotransmitter, a (poly)peptide (derivative), an opiate, an ophthalmicum, (para)-sympaticomimeticum or (para)sympathicolyticum, a protein(derivative), a psoriasis/neurodermitis drug, a mydriaticum, a psychostimulant, rhinologicum, a sleep-inducing agent, a sedating agent, a spasmolyticum, tuberculostaticum, an urologicum, a vasoconstrictor or vasodilatator, a virustaticum, a wound-healing substance, an alcohol abuse preparation, an anticonvulsant, an antineoplastic, an antirheumatic, an appetite suppressant, a biological response modifier, a blood modifier, a bone metabolism regulator, a cardioprotective agent, a cardiovascular agent, a central nervous system stimulant, an enzyme, an agent for

erectile dysfunction therapy, a fertility agent, a gastrointestinal agent, a gout preparation, a hormone, an agent for hypercalcemia management, an agent for hypocalcemia management, an immunosuppressive, a migraine preparation, a motion sickness product, an agent for multiple sclerosis management, a muscle relaxant, a nutritional, an ophthalmic preparation, an osteoporosis preparation, an otic preparation, a parasympatholytic, a parasympathomimetic, a prostaglandin, a psychotherapeutic agent, a respiratory agent, a sedative & hyponotic, a skin & mucous membrane agent, a smoking cessation aid, a sympatholytic, a tremor preparation, a urinary tract agent, a vaginal preparation, a vertigo agent, an inhibitor (antagonist), or any other immunologically active substance (such as an immunomodulator, e.g., bacterial extracts or cell wall components like cholera toxin, heat labile toxin, monophosphoryllipid A, or cytokine inducing agents or hormones like thymosin, thymulin, thymopoietin, or phytoimmunostimulants like extracts from Echinacea root, wild indigo root, white cedar leave tips, or synthetic immunomodulators like quinoline derivatives, synthetic peptides, pyrimidine, lipopeptides, or cytokines or immunosuppressants, and signal transduction inhibitors like cyclosporin A, FK506, FTY720, rapamycin), or a promotor (agonist) of the activity of any of above mentioned agents, or any combination of said active substances. It is preferred that said active ingredient does not itself cross the nasal mucosa in practically meaningful quantity without unacceptable side effects.

In another preferred embodiment of the use or the pharmaceutical composition of the present invention the antigen is derived from a pathogen.

In the context of this invention, the term „pathogen“ refers to an entity which through its presence in or on the body leads to or promotes a pathological state which, in principle, is amenable to or could profit from a preventive, curative or adjuvant immunotherapy.

In a most preferred embodiment of the use or the pharmaceutical composition of the invention said pathogen belongs to the class of extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus*, gram-negative bacteria, such as *Meningococcus* and *Gonococcus* species, species of *Neisseria*, gram negative bacteria, including enteric organisms such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Diphtheria*, *Bordetella Pertussis*, and gram-positive bacteria (e.g.

Bacillus pestis, BCG), particularly anaerobes, such as the *Clostridium* species (e.g. *Clostridium tetani*, *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*), bacteria and viruses, which survive and replicate within host cells, comprising mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes*, retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species (e.g. *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae*, *B. ovis*), the causative agent for cholera (e.g. *Vibrio cholerae*), *Haemophilus* species like *H. actinomycentemcomitans*, *H. pleuropneumoniae*, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases; eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body, which do not necessarily result from microbial infections, also belong in this group.

It is most preferred that the antigen, preferably the pathogen, is used in a purified, or even better in a pure form.

Pathogens causing major infective diseases such as hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, or the causative agent for cholera, *Haemophilus* species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases are particularly preferred as are eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body, which do not necessarily result from microbial infections.

In another preferred embodiment of the use or the pharmaceutical composition of the invention the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.

In further preferred embodiment of the use or the pharmaceutical composition of the present invention the allergen belongs to the class of the inhalation allergens, including but not limited to various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; furthermore, food and drug allergens; contact allergens; injection, invasion or depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., or is a part of implantation material.

In an additional preferred embodiment of the use or the pharmaceutical composition of the present invention said pharmaceutical composition comprises a compound which releases or induces cytokine or anti-cytokine activity or exerts such an activity itself.

The term „cytokine“, as used in the present invention, denotes cytokines, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, with all subtypes, such as IL-1 α and IL-1 β , tumour necrosis factor (TNF), transforming growth factor (TGF- β and - α), Type I and II interferons (IFN- α 1, IFN- α 2, (IFN- ω), IFN- β , IFN- γ), migration inhibitory factor, MIF, c-kit ligand, granulocyte macrophage colony stimulating factor (GM-CSF), monocyte macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), chemokines, etc., as well as all functional derivatives of any of these molecules.

Cytokines that mediate natural immunity particularly well include type I interferons (IFN- α and IFN- β), tumour necrosis factor (TNF), interleukin-1 (IL-1 α and IL-1 β), interleukin-6 (IL-6) and leukocytes attracting and activating chemokines. Antiproliferative (e.g. with IFN-s), pro-inflammatory (e.g. with TNF, IL-1) or co-stimulatory (e.g. with IL-6) action, amongst other, may be generated by transnasal administration of the pharmaceutical composition described in accordance with the present invention. Cytokines which best mediate lymphocyte activation, growth and differentiation include interleukin 2 (IL-2), interleukin-4 (IL-4) and transforming growth factor (TGF). Such cytokines, consequently, not only can affect target growth but, moreover, influence the activation of, and thus the production of other cytokines by, the cells which finally may play a role in therapeutic or prophylactic action.

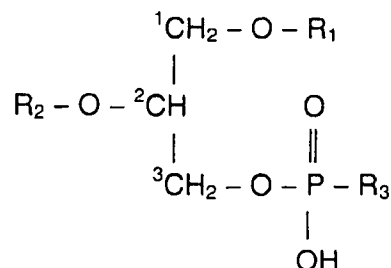
Cytokines that mediate the immune-mediated inflammation which heavily relies on the cell-mediated response are interferon-gamma (IFN- γ), lymphotoxin (TNF- β), interleukin-10 (IL-10), interleukin-5 (IL-5), interleukin-12 (IL-12) and, probably, migration inhibition factor. Leukocyte growth and differentiation are most affected by interleukin-3 (IL-3), c-kit ligand, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage or granulocyte colony stimulating factor (M-CSF or G-CSF) and interleukin-7 (IL-7).

It is preferred to select the compound displaying cytokine activity amongst IL-4, IL-2, TGF, IL-6, TNF, IL-1 α and IL-1 β , a type I interferon, amongst which IFN-alpha or IFN- β are most preferred, IL-12, IFN- γ , TNF- β , IL-5 or IL-10.

In another preferred embodiment said compound with anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative, or an analogue thereof.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention, the compound displaying or inducing cytokine or anti-cytokine activity and the pharmaceutically active ingredient or antigen or allergen are associated with the penetrant, e.g. in the form of a complex, hetero-aggregate, via encapsulation etc.

In an additional preferred embodiment of the use or of the pharmaceutical composition of the present invention the less soluble self-aggregating molecule is a lipid, preferably a polar lipid, and the more soluble component is a surfactant or some more soluble form of the polar/basic lipid. The former ingredient, typically, stems from a biological source or is a corresponding synthetic lipid or any of its modifications. Such lipid often belongs to the class of phospholipids with the chemical formula



where R_1 and R_2 is an aliphatic chain, typically a C_{10-20} -acyl, or -alkyl or a partly unsaturated fatty acid residue, in particular, an oleoyl-, palmitoeloyl-, elaidoyl-, linoleyl-, linolenyl-, linolenoyl-, arachidoyl-, vaccinyl-, lauroyl-, myristoyl-, palmitoyl-, or stearoyl chain, and where R_3 is hydrogen, 2-trimethylamino-1-ethyl, 2-amino-1-ethyl, C_{1-4} -alkyl, C_{1-5} -alkyl substituted with carboxy, C_{2-5} -alkyl substituted with hydroxy, C_{2-5} -alkyl substituted with carboxy and hydroxy, or C_{2-5} -alkyl substituted with carboxy and amino, inositol, sphingosine, or salts of said substances, said lipid comprising also glycerides, isoprenoid lipids, steroids, sterines or sterols, of sulphur- or carbohydrate-containing lipids, or any other bilayer forming lipids, and preferably is selected from the group of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids, phosphatidylserines, sphingomyelins or other sphingophospholipids, glycosphingolipids (including cerebrosides, ceramidepolyhexosides, sulphatides, sphingoplasmalogens), gangliosides or other glycolipids or synthetic lipids, in particular with corresponding sphingosine derivatives, or any other glycolipids, whereby two similar or different chains can be esterified to the backbone (as in diacyl and dialkenoyl compound) or be attached to the backbone with ether bonds, as in dialkyl-lipids, or belong to the backbone as in sphingolipids.

The surfactant used, normally, is nonionic, zwitterionic, anionic or cationic, especially a fatty-acid or -alcohol, an alkyl-tri/di/methyl-ammonium salt, an alkylsulphate salt, a monovalent salt of cholate, deoxycholate, glycocholate, glycodeoxycholate, taurodeoxycholate, taurocholate, etc., an acyl- or alkanoyl-dimethyl-aminoxide, esp. a dodecyl- dimethyl-aminoxide, an alkyl- or alkanoyl-N-methylglucamide, N-alkyl-N,N-dimethylglycine, 3-(acyldimethylammonio)-alkanesulphonate, N-acyl-sulphobetaine, a polyethylene-glycol-octylphenyl ether, esp. a nonaethylene-glycol-octylphenyl ether, a polyethylene-acyl ether, esp. a nonaethylen-dodecyl ether, a polyethylene-glycol-isoacyl ether, esp. a octaethylene-glycol-isotridecyl ether, polyethylene-acyl ether, esp. octaethylenedodecyl ether, polyethylene-glycol-sorbitane-acyl ester, such as polyethylenglykol-20-monolaurate (Tween 20) or polyethylenglykol-20-sorbitan-monooleate (Tween 80), a polyhydroxyethylene-acyl ether, esp. polyhydroxyethylene-lauryl-, -myristoyl-, -cetylstearyl-, or -oleoyl ether, as in polyhydroxyethylene-4 or 6 or 8 or 10 or 12, etc., -lauryl ether (as in Brij series), or in the corresponding ester, e.g. of polyhydroxyethylen-8-stearate (Myrj 45), myristate-, -laurate, linoleate-, linolenate-, palmitoleate- or -oleate type, or in polyethoxylated castor oil 40, a sorbitane-monoalkylate (e.g. in Arlacel or Span), esp. sorbitane-monolaurate, -myristate,

-linoleate, -linolenate-, -palmitoleate- or -oleate, an acyl- or alkanoyl-N-methylglucamide, esp. in or decanoyl- or dodecanoyl-N-methylglucamide, an alkylsulphate (salt), e.g. in lauryl-, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, linolenyl-, linoleoyl-, vaccinyl-, or elaidoyl-sulphate, sodium deoxycholate, sodium glycodeoxycholate, sodium oleate, sodium taurate, a fatty acid salt, with similar preference for aliphatic chains as given above, a lysophospholipid, such as n-octadecylene(=oleoyl)-glycerophosphatidic acid, -phosphorylglycerol, or -phosphorylserine, n-acyl-, e.g. lauryl, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, elaidyl-, vaccinyl-, linoleyl-, linolenyl-glycero-phosphatidic acid, -phosphorylglycerol, or -phosphorylserine, or a corresponding short, double chain phospholipid, such dodecylphosphatidylcholine, or else is a surface-active polypeptide. It is important to realise, however, that complexes of polar lipids with other amphipats often can take the role of surfactants in the coating of a carrier and that different ionisation or salt forms of the polar lipids may differ widely in their properties. It therefore stands to reason that two different physicochemical states of the same (polar) lipid mixed together in a membrane may produce a highly deformable carrier satisfying the conditions of this invention.

In an additional preferred embodiment of the use or of the pharmaceutical composition of the present invention, the more soluble component is an agent to be transported across the barrier, said agent having a tendency to form common large structures with the less soluble component(s) of the penetrant, typically in the form of a physical or a chemical complex.

In a further preferred use or pharmaceutical composition of the invention, the more soluble component tends to solubilise the penetrating droplet and is present in concentration not exceeding 99 mol% of the concentration required to disintegrate the droplet or, alternatively, not exceeding 99 mol% of the saturating concentration in the unsolubilised droplet, whichever is higher, values below 50% of the former relative concentration being particularly useful, with values below 40 rel-% or even around and below 30 rel-% being even more advantageous, whereas in the case of droplets which cannot be solubilised by the more soluble component relative concentrations which exceed the above mentioned relative concentrations by the factor of up to 2 are most preferred.

In a different preferred embodiment of the use or of the pharmaceutical composition of the invention, the less soluble penetrant component is a lipid, preferably a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of polar lipid which is sufficiently soluble for the purpose of this invention.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention, the average penetrant diameter is between 25 nm and 500 nm, preferably between 30 nm and 250 nm, even more preferably between 35 nm and 200 nm and particularly preferably between 40 nm and 150 nm.

In a different preferred embodiment of the use or of the pharmaceutical composition of the present invention the penetrant concentration in the formulation for the use in the human or animal nose is 0.001 weight-% (w-%) to 20 w-% of total dry mass in the formulation, in particular between 0.01 w-% and 15 w-%, more preferably between 0.1 w-% and 12.5 w-% and most preferred between 0.5 w-% and 10 w-%.

In a further preferred embodiment of the use or of the pharmaceutical composition of the present invention the supporting medium, e.g. a buffer, is selected to be a biocompatible solution with an osmotic activity similar to that of a monovalent electrolyte with a concentration range between 1 mM and 500 mM, more preferably between 10 mM and 400 mM, even more preferably between 50 mM and 300 mM, and most preferably between 100 mM and 200 mM or else such solution that affords practically sufficient penetrant stability combined with practically sufficient transport rate across the barrier. The term "practically sufficient penetrant stability" means that the penetrant stability meets the reasonable product quality criteria. The term "practically sufficient transport rate" means that enough drug is transported through the barrier without using unreasonably large application volume or time. Said sufficient penetrant stability combined with sufficient transport rate across the barrier can be determined by the person skilled in the art without undue experimentation.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention, the relative drug or agent concentration is between 0.001 w-% and 40 w-% of total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 25 w-% and most preferably between 0.5 w-% and 15 w-%.

In one further preferred embodiment of the use or of the pharmaceutical composition of the present invention the medium supporting the drugs and carriers is a biocompatible buffer with pH value between 4 and 10, more frequently between 5 and 9 and most often between 6 and 8.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention additives are included in said composition to reduce the system sensitivity to chemical, biological or ambient stress, including anti-oxidants, antagonists of undesired enzyme action, cryo-preservants, microbicides, etc., or else modulators of physically important system properties, such as formulation viscosity, etc.

In a different preferred embodiment of the use or of the pharmaceutical composition of the present invention the relative drug or agent dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.1x and 500x, more often between 0.5x and 250x, and even more preferably between 1x and 100x different from the corresponding drug or agent dose that would have to be injected to achieve the desired biological effects. Again, the latter dose can be determined by the person skilled in the art without undue experimentation and on the basis of his common general knowledge.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention the applied penetrant dose is between 0.01 mg and 15 mg per nostril, even more often is in the range 0.1 mg and 10 mg per nostril, and preferably is between 0.5 mg and 5 mg per nostril.

The efficiency of administration and the biological effects of the agent or drug chosen, consequently, can be controlled by using different application volumes. Various metered delivery devices can be used for the purpose.

Accordingly, in an additional preferred embodiment of the use or of the pharmaceutical composition of the present invention said formulation is administered using a metered delivery device.

In one further preferred embodiment of the use or of the pharmaceutical composition of the present invention different application volumes are selected to control the efficiency of administration and the biological effects of the chosen agent or drug.

In a different preferred embodiment of the use or of the pharmaceutical composition of the present invention the penetrants in suspension are loaded with the drugs or agents within 24 hours prior to the formulation administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the resulting formulation is administered in the nose. This embodiment is expected to improve the formulation stability, loading efficiency, the release kinetics, ease of use, compliance, etc.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention the, delivery device is loaded at the treatment site.

In a further preferred embodiment of the use or of the pharmaceutical composition of the present invention the delivery device is loaded separately with penetrants and the molecules, particularly biological agents, to be associated therewith.

In one further preferred embodiment of the use of the present invention wherein the pharmaceutically active ingredient is for administration to the nervous system.

The term "administration" in connection with this embodiment means that the pharmaceutical composition is transnasally applied, but the target site of the active ingredient is the nervous system, preferably the CNS and most preferably the brain. The possibility to use nasal application of the highly adaptable, drug-loaded penetrants in the nose to mediate a practically useful transfer of the drug across the barrier can thus be exploited to transport a meaningful amount of the drug, and to create a significant concentration of such drug, in the central nervous system or some other adjacent tissue, such as the eye.

In another preferred embodiment of the invention, the pharmaceutical composition of the invention is a vaccine.

Said vaccine can be used for therapeutic or prophylactic vaccination.

The term „(therapeutic) vaccination“ in the context of this invention describes any kind of therapeutic immunisation, whether done after the disease has been already established, to improve a clinical situation, or else for the purpose of preventing a disease. Such a vaccination can involve single or repeated administration(s) of the vaccine of the invention. Therapeutic vaccination will either prevent a pathological situation and/or improve a clinical situation. When applied as a preventive agent, it will generally result in a protective immune response.

Immunisation denotes any kind of provoking an immune response, irrespective of whether said response is therapeutic or non-therapeutic.

An „antibody“ or an „immunoglobulin“ denotes an IgA, IgD, IgE, IgG, or IgM, including all subtypes, such as IgA1 and IgA2, IgG1, IgG2, IgG3, IgG4. Their “derivatives” include chemical, biochemical and otherwise obtainable derivatives, such as genetically engineered antibody derivatives. Fragments include e.g. single chain fragments, Fc-, Fab- F(ab')₂- and other parts of Ig-s, independent of whether they are of endogenous, xenogenic, (semi)synthetic or recombinant origin. Also comprised by the invention are complexes of two or more of the above-recited antibodies, derivatives or fragments.

The term „immunogen“ denotes a hapten coupled to an immunological carrier or an antigen, free or associated with a carrier, which is capable of inducing an immune response.

„Immuno-tolerance“ denotes the lack or, more generally, the reduction of an undesired immune response to an antigen.

Th1 (T-helper cell type I) related antibodies include IgG2a, IgG2b and IgG3.

Th2 (T-helper cell type II) related antibodies comprise the classes of IgG1, IgG4 and IgE.

As has been indicated above, the successful immunisation with the vaccine of the invention through the nose is a significant step forward in the design of conveniently administrable vaccines that (a) are highly efficient over a wide range of immunogens of

varying size and properties; (b) can be formulated together with certain cytokines, compounds that mediate cytokine activity or compounds that antagonize cytokine activity in order to specifically direct the corresponding immune response or to augment or suppress the same as may be desired; (c) do not depend on the perturbing injection by a needle; and (d) cause no irritating side effects. In addition, with the vaccine of the invention, successful tolerogenesis may be achieved.

It has inter alia been found in accordance with the present invention that

- Tween-SPC micelles give protection significantly below that of the vaccine of the present invention, suggesting that the small size of the carrier or the presence of surfactants alone does not suffice for a successful immunisation;
- orally administered immuno-carriers create lower specific antibody titers than the transnasally administered vaccine of the invention, as determined on the basis of absorbance measurements;
- the transnasal vaccine of the invention gives rise to higher specific IgG1 and IgG2 titers in the blood and to comparable IgG2a and IgM titers as compared to mixed micelles; all titers were, on top of this, higher than those generated by immunisation with SPC:cholesterol (1:1) liposomes.

When the transnasal vaccine of the invention is formulated together with a cytokine or an immunoadjuvant it is advantageous to use (blends of) bacterial extracts. Specific examples given in this application include monophosphoryl lipid A (MPL) and IL-12 or GM-CSF and IL-4. In principle, however, the vaccine of the invention may be formulated or applied together with any of the compounds mediating, inducing or displaying cytokine activity or with antagonists thereto that have been recited herein above.

It is preferred that the vaccine of the invention further comprises a pathogen extract or a compound from a pathogen or a fragment or a derivative thereof.

Most preferably, said pathogen extract or compound is selected from hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps or polio viruses, cytomegalovirus, rhinovirus, etc., or fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including

the causative agent for cholera (e.g. *Vibrio cholerae*), *Haemophilus* species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus or rubella diseases.

It is additionally preferred that said vaccine further comprises an adjuvant.

The term „adjuvant“ is used here to describe any substance which supports, augments, stimulates, activates, potentiates or modulates the desired immune response of either cellular or humoral type, specifically in the case of a prophylactic treatment by increasing the antigen specific immune response of any kind and in the case of therapeutic treatment often by supporting cell-mediated immunity. This can be achieved by the addition of suitable cytokines, their blends or suitable agonists and antagonists. The class of immunoadjuvants which indirectly contribute to the useful cytokine pool includes small chemical entities with an allergenic potential, such as certain allergenic (metal) ions, including but not limited to LiCl, HgCl₂, molybdenum, acids, bases and other irritating compounds, such as dicyclohexylmethane-4,4'-diisocyanate, ditrocarb (diethyldithiocarbamate), 2,4-dinitrochlorobenzene, isoprinosine, isophorone-diisocyanate, levamisole, (phenyl)oxazolone and alike, Swansonine, sizofran, phthalic anhydride, thymopentin, (fatty) alcohols, (fatty) amines, (fatty) ethers, ricin, or other suitable amphiphiles, many surfactants and chemical permeation enhancers, as well as derivatives or combinations thereof; furthermore, (low molecular weight) fragments of or derivatives from microbes, including lipopolysaccharides (such as LPS), cord-factor (trehalose-dimycolate) and other (poly)saccharides or (poly)peptides attached to membranes, used in sufficient quantity, acetylmuramyl-alanyl-isoglutamin, and larger fragments of microbes, including bacterial exo- and endotoxins, or enterotoxins, such as cholera toxin and the heat labile toxin of *E. coli*, and their macromolecular fragments, such as A-chain derivatives, most, if not all, of which seem to possess ADP-ribosylating activity, the high potency immunoadjuvant LT holotoxin, etc., cell-wall skeleton, attenuated bacteria, such as BCG, etc. Less established examples include clostridial toxin, purified protein derivative of *M. tuberculosis*, LT-R192G, Fibronectin-binding protein I of *Streptococcus pyogenes*, outer membrane protein of group B *Neisseria meningitidis* (GBOMP), various other peptidoglycans, etc. Immunoadjuvants, in other words, include molecules that alter the uptake or presentation of antigens, activate or increase the proliferation of antigen specific lymphocytes, or interfere with the dominant control mechanism in the immune response, not just in the nose but also in the other

immunocompetent tissues. (The mucosal adjuvant activity of ADP-ribosylating bacterial enterotoxins is a well established and known example for this.) On the other hand, molecules which change the (relative) concentrations of cytokines or other immunoadjuvants, such as anti-immunoadjuvant antibodies or other agonists or antagonists of immunoadjuvants, also are immunoadjuvants in the sense of this invention. The same is true for molecules which affect lymphocyte homing, such as various selectins (LECAMS, e.g. various CD62-s), GlyCAM-1, MadCAM-1, VCAM-1, ICAM-1, hyaluronate, etc., and other chemokines, such as RANTES or MCP-1. Endogenous group of immunoadjuvants furthermore comprises histamines, transfer factor, tuftsin, etc. As many of the above mentioned immunoadjuvants do not have sufficient potency to ensure the desired effect after the non-invasive immunisation at too low, and sometimes too high, concentration or on their own, the functional definition of an adjuvant used in this work includes a fortiori sufficient and such modulation of cytokine concentration and distribution pattern in the body that results in mounting the desired therapeutic or prophylactic immune response. If required to gain clarity said modulation and its extent must be determined in a dedicated experiment, in which the specific cytokine levels are determined, using methods known to the person skilled in the field.

In a further preferred embodiment of the vaccine of the invention, said adjuvant is lipopolysaccharide, such as lipid A or a derivative or modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a microorganism; an extract of a microorganism, including bacterial exo- and endotoxins, preferably cholera toxin or the heat labile toxin of *E. coli*, an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, an LT halotoxin, purified protein derivative of *M. tuberculosis*, LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes*, or outer membrane protein of group B *Neisseria meningitidis* (GBOMP), bacterial or viral nucleic acids, such as oligonucleotides comprising unmethylated CpG dinucleotides.

It is most preferred that the vaccine of the invention comprises a blend of MPL and IL-12 or GM-CSF and IL-4, when pure cytokines and their inducers are used.

In a different preferred embodiment of the vaccine of the present invention the relative immunogen/antigen dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.01x and 100x, more often between 0.05x and 75x, and even more preferably between 0.1x and 50x different from the corresponding immunogen/antigen that would have to be injected to achieve the desired biological effect. Again, the latter dose can be determined by the person skilled in the art without undue experimentation and on the basis of his common general knowledge.

It is further preferred in accordance with the invention that in said vaccine the concentration of the transnasally administered adjuvant is between 10x lower and up to 1000x higher than used with the corresponding subcutaneously injected formulations employing similar antigen, the transnasally administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.

The invention also relates to a container comprising the pharmaceutical composition recited herein-above. The unit dosage may be determined according to the desired application.

Furthermore, the invention additionally relates to a package comprising at least one container comprising the pharmaceutical composition as described above. The package of the invention can comprise one, two, three, four or more vials/units of the pharmaceutical composition of the invention.

The invention finally relates to methods of treating a patient in need thereof comprising transnasally administering any of the above recited pharmaceutical compositions.

The present invention further relates to a method for generating a protective or tolerogenic immune response on a mammal by vaccinating said mammal with a vaccine as described above.

In a preferred embodiment of the method according to the present invention different administration volumes are selected to control the applied immunogen dose and the

outcome of vaccination. Various metered devices can be used for the purpose.

In one more preferred embodiment of the method according to the present invention a suspension of antigen-free penetrants is loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before administering the resulting formulation in the nose.

In another preferred embodiment of the method according to the present invention at least one dose of vaccine is administered.

This embodiment of the method of the invention includes the repeated administration of the vaccine of the invention. Repeated administration includes repeated administration in the nose or one or more administrations in the nose in combination with conventional, e.g. parenteral administrations. In this connection, the kit of the invention may be advantageously made to comprise one or more containers, ampules or other kind of units comprising the vaccine of the invention.

In a particularly preferred embodiment of the method according to the present invention the time interval between the subsequent vaccinations is chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years.

In a further preferred embodiment, repeated immunogen administration is advocated to maximise the final effect of a therapeutic vaccination. It is proposed to use between 2 and 10, often between 2 and 7, more typically up to 5 and most preferred up to 3 immunisations, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined as described above or by some other suitable assessment method, or else to deem the effort as having failed. The time interval between subsequent vaccinations should preferably be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years, when a subject is being immunised for the first time. Rodents, such as mice and rabbits are advantageously immunised in 2 weeks interval, primates, e.g. monkeys and often humans, need a booster vaccination in 3-6 months interval.

In another preferred embodiment of the method according to the present invention the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is determined as a function of the suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further.

The disclosure content of the documents cited throughout this specification are herewith incorporated by reference. Further incorporated by reference is the complete disclosure content of the co-pending application filed in the name of IDEA AG and bearing the title "Noninvasive vaccination through the skin".

The figures show:

Figure 1 illustrates the effect of nasal insulin administration by means of carriers in an insulin dependent diabetes mellitus patient, with the result of an i.v. injection of fast-acting insulin (Actrapid, Novo-Nordisk) shown in the inset for reference.

Figure 2 illustrates the glucodynamics in a healthy human volunteer following intranasal administration of insulin by means of Transfersomes. Inset gives the result of intravenous injection of similar formulation for reference purpose.

Figures 3a and 3b provide further examples measured with a healthy volunteer following intranasal administration of insulin formulations with inferior characteristics, believed to be due to too slow drug release from the carrier.

Figure 4 illustrates the capability of nasally administered cytokines, associated with Transfersomes, to affect the outcome of transnasal immunisation with tetanus toxoid.

Figure 5 illustrates the biodistribution of insulin-derived radioactivity in mice following nasal administration of the agent in transfersomes.

Figure 6 gives the corresponding results for interferon, as measured in mice.

Figures 7 illustrate the effect of changing aggregate size and/or deformability on TT specific immune response in mice treated with various mixed micelles, Transfersomes or liposomes loaded with TT. Panels a and b show antibody isotype patterns, and in panel c the total antibody titre, as expressed in absorbency change is given.

Figures 8 highlight the (small) effect of changing antigen dose (in the high dosage range) on transnasal immunisation of mice with TT by means of Transfersomes with or without lipid A derivative as an immunoadjuvant. In panel a, the results of total absorbance measurements are given, panel b shows the corresponding titration curves, and panel c gives the relevant antibody isotypes.

Figure 9 is organised in similar fashion to compare the outcome of intranasal, oral or subcutaneous TT administration using different antigen doses and purity.

Figure 10: For comparison, animal protection (survival) data are given for the experiments in which several doses and administration routes were compared.

Figure 11 presents a set of data on the effect of various cytokines, or their combination, on the murine immune response to TT administered into the nose by means of transfersomes, with s.c. data given for comparison. Panel a gives the absorbance and titre data and panel b contains the isotype distribution results.

Figure 12 deals with the effects of combining low and high molecular weight immuno-adjuvants (lipid a analogue and interleukin-12).

Figure 13 illustrates the effect of specific cytokine inducers of microbial origin. Cholera toxin (CT) is used for the purpose.

Figure 14 shows one effect of heat labile toxin from E. coli as immuno-adjuvant.

Figure 15 illustrates the results obtained with a combination of two antigens, tetanus toxoid and cholera toxoid.

The examples illustrate the invention.

EXAMPLES

General experimental set-up and sample preparation

Conventional vesicles, liposomes, comprised soy phosphatidylcholine (SPC; Nattermann Phospholipids, Rhone-Poulenc Rorer, Cologne, Germany).

The suspension containing 10 w-% of the lipid in form of multilamellar vesicles was prepared by suspending the lipid in a buffer and then extruding the suspension through several polycarbonate membranes (with 800 nm, 400 nm, 200 nm and 100 nm pores, respectively) to narrow down the final vesicle size distribution. If required, as judged on the basis of optical inspection or the dynamic light scattering done after the latter steps, extrusions were repeated several (up to 5) times. In some cases, the vesicles were first extruded to a diameter of app. 50 nm and then frozen and thawed three times to enlarge the vesicles again, owing to inter-vesicle fusion. Subsequently, the formulation was passed through a micro-porous filter (100 nm; Poretics, CA), under pressure, to prepare the final suspension of oligo- or unilamellar vesicles.

Highly adaptable penetrants, used in the described examples, typically had the form of ultradeformable vesicles (Transfersomes™) with one or a few bilayers. They comprised a mixture of phosphatidylcholine and (bio)surfactants (cholate or polysorbate (Tween 80)), and various biologically active ingredients, such as insulin, interferon, interleukin, or GC-SF.

The above mentioned penetrants were prepared by mixing the phospholipid(s) with a suitable membrane-softening agent, such as cholate or polysorbate, as the case may be, either in an aqueous buffer or in ethanol; occasionally chloroform was used. In the latter two cases, which gave similar results, the solvent was evaporated under vacuum (10 Pa, overnight). The resulting lipid film was then hydrated with a buffer (pH around 7) to get a 10 wt-% lipid suspension, by and large. Vesicles were brought to the final, desired size by sequential extrusion as described for liposomes, using mainly filters with smaller pore sizes. The final size of Transfersomes was similar to that of liposomes.

Changing the surfactant-to-lipid ratio is believed to affect the mixed lipid bilayers deformability: the higher the surfactant concentration, the more adaptable is the resulting aggregate, up to the concentration at which the mixed lipid membranes became unstable, owing to the high surfactant concentration. At such point the mixed aggregates revert into micelles which no longer change their shape easily, owing to the low compressibility of the micelle interior. Vesicles without a surfactant or some other edge active ingredient, which are commonly known as liposomes and have at least 10x less flexible membranes than the more deformable mixed lipid vesicles, are a convenient negative control for the latter. The other obvious control are

Mixed lipid micelles containing similar ingredients as the corresponding highly adaptable penetrants, but in a different ratio, such that the edge active component (typically, but not necessarily, the surfactant) concentration is above the solubilization concentration value. To prepare said micelles, individual components were mixed in the aqueous phase and permitted them to interact until the mixture became optically clear, that is, solubilised, as judged by optical inspection or absorption measurement at 400 nm to 600 nm.

Experiments carried out on human volunteers

To test biological activity of insulin carriers in humans, a freshly prepared test formulation was used in the nose of two test subjects. The first was a normoglycaemic (male, 74 kg, 173 cm, 45 years); the second was a C-peptide negative IDDM patient (female, 62 kg, 167 cm, 26 years). The test persons fasted between 6 h and 12 h prior to insulin administration.

To follow the temporal variation of glucose concentration in the blood, 5 μ L to 30 μ L samples taken, every 10 min to 15 min, from the fingers on both arms. After an initial test period, during which the 'normal' blood glucose concentration and/or its change was determined, a suspension of carriers loaded with insulin (Transfersulin) was sprayed into each nostril, using conventional non-metered sprayer, in a series of 150 μ L puffs. Care was taken to minimise the spill-over of test formulation into the throat or the dropping of said formulation from the nose.

Commercial glucometer (Accutrend™, Boehringer-Mannheim) was employed to determine the blood sugar concentration. At each time point, three individual, independent readings were made, except when the standard deviation was so high as to require repeated measurements.

The test formulations were made essentially as described in patent application PCT/EP98/06750. In brief, a suspension of highly adaptable penetrants with the above mentioned composition and an average diameter of the order of 100 nm to 150 nm was loaded with the drug, based on interfacial adsorption, and used within 24 h after the preparation. The drug-carrier association in the formulation was determined to be between 60% and 70%.

To administer the drug laden suspension into the nose, the preparation was filled into a commercial nebuliser (with a hand-driven pump, vertically oriented spraying nozzle and a puff volume of 150 μ L, on the average). One puff was given into each nostril at a time, while the test subject gently sniffed.

The total number of puffs was a function of the application dose (in this case: 2). Immediate spill-over into the throat or partial leakage of the fluid from the nose was reported in 10-20% of cases. No side effects, such as local irritation, sneezing, etc., were observed.

Example 1:

28.4 mg/mL phosphatidylcholine from soy-bean
9.5 mg/mL phosphatidylglycerol from soy-bean
62.1 mg/mL Tween 80
phosphate buffer, pH 7.4
human recombinant (hr) insulin, 50 IU/mL
(from Actrapid 100 HM™, Novo-Nordisk)

Applied dose: ~5 IU per nostril

Results measured with a healthy subject are shown in figure 1. They reveal a transient decrease in the systemic blood glucose concentration after two administrations of the

drug in carriers (closed symbols), with a maximum after 20-30 min and a return to the pre-treatment value after approximately 1 h in either case. The observed change in glucose level corresponds to approximately 8.5% of the decrease was measured in an independent experiment after intravenous injection of the drug (Inset: open symbols). The reproducibility remains to be improved, however, the first application, biased by the lack in administration skill having been less successful than the second administration.

No irritation or other unpleasant sensation was reported by the test person after nasal administration of insulin in highly adaptable penetrants.

Example 2:

Insulin loaded, highly adaptable carriers in an IDDM patient

highly adaptable penetrants:

as given in example 1

Applied dose: 25 IU per nostril

Test preparation and experiment was performed as described with previous example. The last administration of conventional insulin (Monotard™, Novo-Nordisk), at the dose of 22 IU was done at 10 p.m. on the previous day. Test subject, moreover, was stabilised by using long-acting insulin on the test day prior to nasal administration of the insulin associated with highly adaptable drug carriers.

Results of an experiment done with said IDDM patient is illustrated in figure 2. Owing to the lack of endogenic insulin production in this test subject, the pre-treatment blood glucose concentration was slightly above the normal, but relatively constant. The change resulting from nasal drug administration with ultra-adaptable carriers, has more a step-like rather than a peak-like shape (closed symbols), completed within 75 min. This is precisely what one would expect for an IDDM patient. The result of an i.v. injection of rapidly acting insulin (Actrapid™, Novo-Nordisk) in the same test person on a different occasion (inset: open symbols) corroborates the conclusion. An estimate of

apparent bioavailability of nasal insulin based on these data is around 4% and, consequently, somewhat lower than that reported in example 1. This may have to do with the presumed variability in drug release between different formulations which is illustrated in the following examples.

Nasal administration of carrier-associated insulin, according to the test person, caused no adverse side effect, locally or systemically.

Examples 3-5:

Insulin associated with suboptimal carriers

Carriers

as in previous examples, but believed not to release the drug readily owing to the higher affinity of selected insulin batch for the carrier, which makes the drug adsorption irreversible.

Applied doses: 50 IU, 50 IU

Results of the test measurements done with several different vesicle suspensions, illustrated in figure 3, signal lack of action for the insulin administered nasally with such carriers. The blood glucose concentration in the investigated normoglycaemic test person remains the same before, during and after the drug administration, for several hours at least. This suggests that the mere presence of carriers, or their ingredients, is insufficient to improve the bioavailability of nasally applied macromolecules, such as insulin. To achieve the desired biological effect, the rate of drug release from the carrier must also be adequate, such rate being determined in dedicated ex vivo experiments by using conventional protein binding deassociation techniques.

Animal experiments

Examples 6-9 :

Labelled insulin delivery across nasal mucosa of test mice

highly adaptable penetrants:

87.4 mg/mL phosphatidylcholine from soy bean (SPC)

12.6 mg/mL of a 50 % ionised cholic acid

phosphate buffer, 50 mM, pH 6.5

hr-Insulin (Actrapid™, Novo-Nordisk)

labelled insulin from Amersham

(345 μ L contain 1.08 μ g insulin and 1.725 mg BSA)

¹²⁵I-labelled insulin (210 μ L) was mixed with 210 μ L of hr-insulin (Actrapid™ Novo-Nordisk, 100 HM) and purified 2 times by centrifugation to eliminate the non-bound label, which diffuses across the barrier much faster and better than whole drug molecules. 100 μ L of the resulting solution was mixed with 150 μ L phosphate buffer to yield pH around 7. Protein solution and lipids were processed together, bringing the final vesicle size by repeated extrusion through 100 nm pore filters to values around 150 nm.

Mice of NMRI strain (36 g to 51 g) from a local supplier were kept in suspension cages in groups of 4 to 6. The animals had free access to standard chow and water. Each mouse received 2.5 μ L of labelled penetrant suspension containing insulin per nostril. Then, the decrease in total radioactivity was assessed by whole-body camera at least 2 times. At different times the mice were killed and all major organs were taken and measured separately. The carcass was measured in two steps, after organ elimination and then after separation of the head. Radioactivity in excrement and cage was also determined.

Results pertaining to different time-points are given in Figure 4. They show that substantial amount of nasally administered radioactivity is recovered from the body, even after exclusion of gastro-intestinal tract, especially during the first hours following

suspension administration. Values in the blood are in the range of 9% at 0.5 h and 2%, the specific concentration falling from 3%/mL at the beginning to 0.7%/mL at the end. Activity in the nose decreases from 10.4% at 0.5 h to 0.3% at 8 h. Liver values are between 2.3% after 0.5 h, the maximum around 2.8 at 1 h and values above 1% after 4 h. After 8 h, the residuum in the liver is around 0.4%. The relatively high hepatic values are suggestive of passage of particles, that is, penetrants, through the barrier and subsequent uptake in the reticulo-endothelial system.

Corresponding CNS values are 0.1% and 0.03%. Maximum in the brain is measured between the first and second hour with app. 0.11% and 0.14%, respectively, which amounts to around 0.3%/g organ. These, apparently low values compare favourably with the result of more conventional drug delivery into CNS which yields values below 0.5% of injected dose or around 0.15%/g organ, for example, when transferrin-receptor is used to deliver the drug (Pasechnik & Price, 1996). In the case of white-germ agglutinin 0.1% was found in olfactory bulb.

Examples 10-11 :

highly adaptable penetrants

87.4 mg/mL phosphatidylcholine from soy bean (SPC)

12.6 mg/mL of a 50% ionised cholic acid

phosphate buffer, 50 mM, pH 6.5

human recombinant insulin (Actrapid™, Novo-Nordisk)

labelled insulin from Amersham

In a related experiment, 345 μ L of 125 I-labelled insulin was mixed with 345 μ L of cold Actrapid™ (Novo-Nordisk) and purified 2 times, as in previous experiment. After addition of 200 μ L phosphate buffer, 150 μ L of resulting solution was mixed with the lipids and extruded to final vesicle size. The applied dose was 3 μ L per nostril. Mice were killed after 1 h, fixed, cut in thin sections and inspected by the whole-body radiography. Free insulin in solution was used for comparison.

The results of above mentioned experiments (not shown) revealed high label accumulation in the nasal region, as one would expect, substantial spill-over into the GI

tract, very high density in the bladder, but also some radioactivity in the liver, which appears to be slightly higher for the carrier-derived than for the free insulin.

Examples 12-13:

Labelled interferon-gamma delivery across nasal mucosa of test mice

highly adaptable penetrants

86.6 mg/mL phosphatidylcholine from soy bean (SPC)

13.4 mg/mL Na cholate

phosphate buffer, 10 mM, pH 7.2 (nominal)

1 mg IFN-gamma/mL suspension

(100 μ Ci/mL suspension, as 3-¹²⁵I-tyrosyl-IFN-gamma)

Applied dose: 5 μ L nostril

Mice of NMRI strain (36 ± 0.6 g) were housed and taken care of as described with previous examples. Prior to the test formulation application, the animals were sedated as described before. Test formulation then was administered through a fine catheter in two drops of 5 μ L, resulting in the total dose of 1 mg lipid. After this, the animals were kept in separate cages to prevent mutual contamination.

Measured radioactivity in the blood was found to correspond to app. 2.5% of the applied dose, liver concentration being at app. 2% and colon concentration around 2.5%, all after 2 h. The highest amount of radioactivity by then was recovered from the stomach (37%) and in the cage plus excrement (32%).

In the central nervous system (CNS) 0.06% of total nasally applied dose, as judged by derived radioactivity, was present 1 h after the drug administration by means of highly adaptable, protein-loaded mixed lipid-surfactant vesicles.

Examples 14-19:

Cytokine delivery across the nasal mucosa of test mice

highly adaptable penetrants

37.7 mg/mL phosphatidylcholine from soy bean (SPC)

62.3 mg/mL polysorbate (Tween 80)

phosphate buffer, 10 mM, pH 6.5

Tetanus toxoid, as antigen (2 mg/mL)

Interferon- γ (IFG- γ)

Granulocyte-monocyte-colony stimulating factor (GM-CSF)

Interleukin 4 (IL-4)

Interleukin 12 (IL-12)

Applied dose: 3 μ L per nostril

Mice of Swiss albino strain (18-20 g) were obtained from The National Institute of Nutrition (Hyderabad, India). They were 8 to 12 weeks old at the time of first immunisation. The antigen alone or in combination with various cytokines, both believed to be at least partly associated with the carriers, was positioned with a sequencing in front of the animal nose and left to be sucked-in by the latter. Blood samples were collected retro-orbitally and tested with specific antibodies directed against the employed antigen by measuring absorbance at 492 nm, after subtraction of blank samples with ELISA.

The results of above mentioned measurements, illustrated in figure 6, suggest that the presence of all tested cytokines in vaccination formulation, based on the highly adaptable antigen carriers, increases the serum absorbance compared to that characterising the non-modulated value, determined after simple immuno-carrier administration. Relative differences are more likely consequences of diverse bio-potency of tested immuno-modulants employed in the present specific experimental system than indicative of variable macromolecular transport rate across the nasal mucosa.

The observed 100% increase in serum absorbance measured for GM-CSF/IL-4 combination is remarkable, as it is known that neither polysorbate nor phosphatidylcholine ex soy-bean can markedly enhance permeation capability on their own. It is therefore reasonable to assume that the observed effect is not simply due to the delivery of antigen molecules (with the molar mass of 150 kDa) across the nasal

mucosa but, moreover, testify that at least a proportion of co-administered cytokines has passed the barrier in a biologically active form.

Examples 20-21:

highly adaptable penetrants

as in examples 14-19, except for the absence of cytokines

Tetanus toxoid antigen (2 mg/mL)

Mixed lipid micelles

14.8 mg/mL phosphatidylcholine from soy bean (SPC)

85.2 mg/mL polysorbate (Tween 80)

phosphate buffer, 10 mM, pH 6.5

Tetanus toxoid antigen (2 mg/mL)

Applied dose: 3 μ L per nostril

Experiments were done as described with previous examples (14-19).

Immune response in the animals treated with mixed lipid micelles as in Examples 14-19 was clearly inferior to that measured after the nasal application of antigen in the highly adaptable lipid vesicles, despite the fact that the latter contained a smaller amount of Tween 80 than the former. If the surfactant was responsible for the transport of macromolecules across nasal mucosa, owing to its action as skin permeation enhancer, precisely the opposite experimental outcome would have been expected.

This suggests that highly adaptable carriers (mixed lipid vesicles) transport macromolecules across the nasal mucosa by a mechanism other than the drug permeation.

Examples 22-29:Aggregate size (stability) effect

Highly deformable vesicles with NaCh (Transfersomes™)

89.3 mg phosphatidylcholine from soy bean

10.7 mg sodium cholate (NaCh)

0.9 mL phosphate buffer, pH 6.5

(Mixed lipid) Micelles with NaCh, type 1

65 mg phosphatidylcholine from soy bean

35 mg sodium cholate

0.9mL phosphate buffer, pH 6.5

(Mixed lipid) Micelles with NaCh, type 2

31.6 mg phosphatidylcholine from soy bean

68.5 mg sodium cholate

0.9 mL phosphate buffer, pH 6.5

Highly deformable vesicles with Tw, Transfersomes™ type 1

37.7 mg phosphatidylcholine from soy bean

62.3 mg Tween 80 (Tw)

0.9 mL phosphate buffer, pH 6.5

Highly deformable vesicles with Tw ,Transfersomes™, type 2

64.5 mg phosphatidylcholine from soy bean

35.5 mg Tween 80

0.9 mL phosphate buffer, pH 6.5

(Mixed lipid) Micelles with Tw, type 1

13.2 mg phosphatidylcholine from soy bean

86.8 mg Tween 80

0.9 mL phosphate buffer, pH 6.5

(Mixed lipid) Micelles with Tw, type 2

7 mg phosphatidylcholine from soy bean

93 mg Tween 80

0.9 mL phosphate buffer, pH 6.5

0.10

Lipid vesicles (liposomes)

65 mg phosphatidylcholine from soy bean (SPC)
35 mg cholesterol
0.9 mL phosphate buffer, pH 6.5

Tetanus toxoid (2 mg/mL; home made) used at the dose of
40 μ g (20 μ L) TT per mouse and immunisation

The medium filtrate from a culture of *Clostridium tetani* grown in vitro was used as an purified antigen. Pure toxoid was purchased from Accurate Antibodies, NY, USA.

To test the effect of aggregate properties in the formulation, three kind of aggregates were prepared: relatively large vesicles (diameter between 100 nm and 200 nm) either comprising a flexible membrane (Transfersomes) or a relatively rigid membrane (liposomes) and much smaller micelles (diameter below 50 nm). The latter were chosen to mimick the more conventional approach of using detergents as nasal mucosa permeation enhancers.

Amongst the eight tested formulations, Transfersomes, on the average, give best results, but absolute titres are always very low, probably owing to the antigen impurity. Mixed lipid micelles are most efficient in creating IgA, but are not really different than the other aggregates in the case of IgG2a and IgM, whilst in the case of IgG2b they are comparable to Transfersomes. The IgG1 level, which is decisive for animal protection, is only significantly elevated when Transfersomes are used to deliver TT across through the nose (see figure 7a).

Mixed micelles containing less potent detergents (with lesser skin permeation enhancing capability) are, relatively speaking, less efficient 'immuno-carriers' (see figure 7b), the more deformable Transfersomes with a higher Tw content standing clearly out in the case of IgG2a and IgM, are similar to the less deformable Transfersomes with a lower Tw content in the case IgG1 and IgG3, and are as efficient as mixed micelles with Tw in the case of IgA and IgG2b. The smallness of measured values is reason for the concern, however, which can best be overcome by using purer antigen.

Looking at the cumulative titre of all specific anti-TT antibodies in the serum, liposomes are relatively efficient 'immuno-carriers' in the primary and mature response (perhaps owing to the action of non-associated TT), whilst the Tw rich mixed micelles are the worst. NaCh Transfersomes are top performers in the late immune response (cf. figure 7c).

Examples 30-35:

Antigen dose and purity effect

Highly deformable vesicles (Transfersomes):

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

+/- 0.04 mol-% monophosphoryl Lipid A (LA) relative to SPC

0.9 mL phosphate buffer, 10 mM, pH 6.5

Tetanus toxoid (TT, from local source, purified by ultrafiltration)

0 μ g, 40 μ g or 80 μ g TT/ mouse/ immunisation

To obtain partially purified antigen, such filtrate was passed through a 10 kDa cut-off membrane and washed thoroughly with phosphate buffer, pH 6.5; in the process, the culture filtrate was concentrated 15 times.

Dose dependence results are illustrated in figure 8a. The TT-specific increase in serum absorbance following TT administration through the nose by means of Transfersomes reveals a positive dose dependence in the primary and late immune response in the absence of LA, the presence of LA reverting this trend. Titre-wise and with regard to specific antibody isotype distribution, similar but not identical picture is obtained (cf. figures 8b and 8c). The survival data are indicative of good protection in every case. Taken together this suggests that the required dose for non-invasive nasal immunisation by means of highly deformable carriers is much lower than that required for a successful non-invasive TT administration through the skin.

Antigen purity effect. Comparison of the data shown in figure 8c and 7a and 7b shows that antigen purity strongly affects the level of murine immune response against tetanus toxin when the toxoid has been applied non-invasively through.

Examples 36-46 :

Route of administration

Highly deformable vesicles, NaCh Transfersomes™

as described with examples 1-8

Tetanus toxoid mixed with NaCh suspension

20 mg/mL sodium cholate in
phosphate buffer, pH 7

Tetanus toxoid dose: 40 µg TT per immunisation; 5 µg TT, 10 µg TT, 20 µg TT, 40 µg TT per immunisation.

Using the same experimental procedures as described with previous examples, the antibody-specific serum absorbance the corresponding antibody titre and isotype distribution, and the level of animal protection against tetanus toxin was determined after nasal, oral and subcutaneous antigen administration.

The results are given in figures 9. They reveal that the increase in serum absorbance, ultimately, is comparable after invasive and non-invasive antigen administration (figure 9a). However, the titre in the latter case is significantly lower except in the primary response. Interestingly, s.c. injection only produces superior results after the second boost, whereas the combination with TT and cholate, which then can act as nasal permeation enhancer in total antibody titre is better at all times. The probable reason for this is the high concentration of IgG2b, as is seen from figure 9b. Injections elicit most efficiently the IgG1 and IgM type of antibodies.

Animals are well protected by any of above mentioned vaccinations with TT, but only after 2 boosts; in the case of nasal vaccination. In contrast, one boost is sufficient

(data not shown). Using 4-8x lower doses of purified TT suffices for protection in the case of nasal vaccination, but not when the antigen is injected (cf. figure 10).

Example 47

Low molecular weight adjuvant (lipid A) effect

Highly deformable immuno-modulated TT-Transfersomes™:

as in examples 9-14

Tetanus toxoid: 2 mg/mL, with 20 μ L or 40 μ L corresponding to 40 μ g or 80 μ g TT per immunisation

It is believed that co-administration of immuno-active, typically immunopotentiating, molecules is advantageous for presentation TT associated with the carriers to the body. To substantiate this conclusion specifically the outcome of non-invasive immunopresentation of TT was compared by means of Transfersomes with or without monophosphoryl lipid A (LA), which is a well known immunostimulant known to elicit generation of TNF, for example. For the used, relatively high antigen doses no strong dependence was found, however. In either case substantial titres and a prophylactic immune response was reached, which was not the case with purified TT which profited from the presence of LA.

Examples 48-53:

High molecular weight immunomodulators (cytokines)

Highly deformable vesicles, Tw Transfersomes™:

as described with examples 1-8, plus

various cytokines (Interferon- γ , GM-CSF, IL-4, IL-12)

(0.05 mg IFN- γ ; 0.004 mg GM-CSF; 0.004 mg IL-4 per mL, 0.004 mg IL-12 per mL)

Tetanus toxoid, 2 mg/mL, corresponding to 40 μ g TT (purified, home prepared) per mouse/ immunisation

The effect of cytokines was studied individually and in combination. The results are given in figures 5. They suggest that GM-CSF plus IL-4 combination can support the generation of anti-TT antibodies in mice, as can, probably, IFN- γ and perhaps IL-12, and maybe IL-4 (cf. figure 11a). The strongest effect is seen in the case of IgM and IgA, except in the case of IL-12 usage, which only affects strongly IgG2b generation. The protection relevant IgG1 is increased strongly only by the combination of GM-CSF and IL-4, whereas IgG3 is not affected at all. Injection works best for IgG1 (cf. figure 11b).

Examples 54-58:

Combination of low and high molecular weight adjuvants (LA + IL-12) effect

Highly deformable vesicles, NaCh Transfersomes™,
as described with examples 1-8, plus
0.4 mg IL-12 per mL immunogen suspension
0.04 mol-% monophosphoryl Lipid A (LA) relative to SPC

Tetanus toxoid (purified), 2 mg/mL, corresponding to 40 μ g TT per mouse/
immunisation

The effect discussed with examples 25-31 was confirmed for a blend low molecular and high molecular weight immunoadjuvants. The results are given in figures 12 and show that the immunopotentialisation by such a combination is especially strong during the early stage of immune response, the combination in any case being better than LA alone.

Examples 59-71:

Bacterial wall component, cholera toxin, as adjuvant:

Highly deformable vesicles, Transfersomes™ (Tfs):

TfsC

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

0.1 mL ethanol

TfsT

36 mg phosphatidylcholine from soy bean (SPC)

64 mg Tween 80

0.9 mL phosphate buffer, 10 mM, pH 7

Cholera toxin (CT; Sigma, Neu-Ulm), 2 μ g/immunisation, if specified,

Tetanus toxoid (TT, pure; Accurate Antibodies), 2 mg/mL.

Volume doses corresponding to 0 μ g TT/mouse/immunisation (negative control), 1 μ g TT/mouse, 5 μ g TT/ mouse, 10 μ g TT/ mouse, 20 μ g TT/mouse, 40 μ g TT/mouse (in the absence of CT) and 0.5 μ g TT/mouse/immunisation, 1 μ g TT/mouse, 5 μ g TT/ mouse (when using CT) was used intranasally in the type T Transfersomes (TfsT) in both nostrils and at the dose of 0.5 μ g TT/mouse/immunisation in the type C Transfersomes (TfsC) in 4-6 Swiss albino mice. Moreover, 20 μ g TT/ mouse/immunisation in TfsT was injected subcutaneously at the corresponding site in the positive control group. Immunisations were done on days 1, 14, 28.

The protective effect of antigen applied in the nose was good when the antigen dose exceeded 20 μ g/immunisation; lower doses yielded insufficient, but detectable protection (cf. figure 13). When cholera toxin (CT) was included into the test formulation together with the tetanus toxoid, excellent protection was achieved already at the lowest of tested doses (0.5 μ g/immunisation), independent of the ultra-deformable carrier composition. Protection was complete in all test groups containing CT in the formulation applied on the skin.

Examples 72-74:

Heat labile toxin from E. coli (HLT) as immuno-adjutant

Highly deformable vesicles, Transfersomes™:

TfsC

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

Heat Labile Toxin (HLT, SIGMA, Neu-Ulm), ≤ 1 mg/mL plus, if required

Tetanus Toxoid (TT, pure, Accurate Antibodies) 2 mg/mL

or

IfsT

36 mg phosphatidylcholine from soy bean (SPC)

64 mg Tween 80

0.9 mL phosphate buffer, 10 mM, pH7

Heat Labile Toxin (LT, SIGMA, Neu-Ulm), ≤ 1 mg/mL, if required,

Tetanus Toxoid (TT, pure, Accurate Antibodies) 2 mg/mL

Antigen carriers were prepared and all experiments (with Swiss albino mice) and assays were carried out as described in the previous examples. A series of different HLT doses ranging from approx. 50 ng/application to multi-microgram quantity per application was used in combination with TT concentrations in the range of approx. 100 ng and up to 10 μ g per application. In most cases, volume doses corresponding to 0.5 μ g TT / mouse / i.n. immunisation and 0.1 to 0.5 μ g HLT / mouse / i.n. immunisation and a positive control with 0.5 μ g TT for s.c. injection was used for mice immunisation.

As is shown in Figure 14, anti-TT titres are improved by HLT acting as an adjuvant in comparison with the result of s.c. injection without an adjuvant. The humoral response is dose dependent, i.e. higher anti-TT titres are achieved with the higher dose of HLT. The correlation is not linear, however, but shows rather a maximum (data not shown).

Protection against tetanus toxin challenge is equally efficient for the high and the low dose of immuno adjuvant.

This suggests that adjuvants should be used in the nose in conjunction with Transfersomes at maximum at the dose that is in the low-end range of doses used for conventional invasive (s.c.) immunisations; the minimum dose for immuno-Transfersomes usage in the nose should also be 1-2 orders lower, for the adjuvant tested in this series for most, if not all, immunoadjuvants.

Example 75:Bivalent vaccination with Tetanus Toxoid and Cholera Toxin as antigens

Highly deformable vesicles, Transfersomes™:

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

Cholera Toxin (CT, SIGMA, Neu-Ulm), ≤ 1 mg/mL

Tetanus Toxoid (TT, pure, accurate antibodies) 2 mg/mL

or

TfsT

36 mg phosphatidylcholine from soy bean (SPC)

64 mg Tween 80

0.9 mL phosphate buffer, 10 mM, pH7

Cholera Toxin (CT, SIGMA, Neu-Ulm), ≤ 1 mg/mL

Tetanus Toxoid (TT, pure, accurate antibodies) 2 mg/mL

Details on the formulation and its preparation, on vesicle characterisation and on animal experiments as well as the following assays are given in the description of related examples hereinabove.

The main result of this experimental series was that Cholera Toxin, added to the formulation as an adjuvant, can also induce the formation of anti-CT antibodies in practically relevant quantity. This effect can, but does not have to, be achieved using concentrations of CT and TT sufficient to ensure good protection against the challenge with tetanus toxin. Figure 15 compares anti-TT and anti-CT titres from the same mice immunised with TT and CT within the same carrier. This reveals the potential of Transfersomes™ containing more than one antigen to serve as the basis for at least bivalent vaccines.

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CLAIMS

1. Use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in the solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, such droplets then acting as carriers for the transnasal administration of a pharmaceutically active compound, an antigen, an allergen, a mixture of antigens and/or a mixture of allergens.
2. Use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in the solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or forms of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to

solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains as a carrier for the preparation of a pharmaceutical, preferably a vaccine composition for transnasal administration.

3. Use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in the solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, said penetrant being used in combination with a pharmaceutically active ingredient or an allergen or an antigen for the preparation of a transnasally administerable pharmaceutical composition for the treatment of infective diseases, endocrine disorders, preferably hypopituitarism, diabetes, hyperthyroidism, thyroiditis, most preferably Hashimoto's thyroiditis, subacute thyroiditis; adrenal disorders, preferably Addison's disease, secondary adrenal insufficiency, Cushing's syndrome; gastrointestinal disorders, preferably Crohn's disease, colitis; hemorrhagic diseases, preferably hemophilia, leukopenia, hypereosinophilic syndrome; musculoskeletal and connective tissue disorders, preferably rheumatoid arthritis, Sjögren's syndrome, Bechet's

syndrome, lupus, scleroderma, polymyositis/dermatomyositis, polymyalgia rheumatica and temporal arthritis, polyarteriosis nodosa, Wegener's granulomatosis, mixed connective tissue disorder, ankylosing spondylitis, psoriatic arthritis, osteoarthritis, Paget's disease, sciatica, bursitis, tendonitis and tenosynovitis, epicondylitis, fibromyalgia, eosinophilic fasciitis; neurological disorders, preferably pain, singultus, vertigo, seizure disorders, sleep disorders, transient ischemic attacks, spinal cord injury, demyelinating diseases, nerve root disorders, myasthenia gravis; oncological disorders; psychiatric disorders, preferably drug dependence, neuroses, mood disorders, schizophrenic disorders, delusional disorders; and/or for use in the field of gynecology, preferably for the treatment of dysmenorrhea, menopause, chronic anovulation, premature ovarian failure, endometriosis, infertility; and/or for use in the field of immunology, preferably transplant rejection, hyposensitisation, allergen immunotherapy or prophylactic vaccination.

4. A pharmaceutical composition for transnasal administration comprising a carrier, which is a penetrant, suspended or dispersed in an aqueous solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains said composition also including a

pharmaceutically active ingredient, an allergen, an antigen, a mixture of antigens and/or a mixture of allergens.

5. The use of claim 3 or the pharmaceutical composition of claim 4 wherein the pharmaceutically active ingredient is an adrenocorticostaticum, an adrenolyticum, an androgen or antiandrogen, an antiparasiticum, an anabolicum, an anaestheticum or analgesicum, an analepticum, an antiallergicum, antiarrhythmicum, antiarteroscleroticum, antiasthmaticum and/or bronchospasmolyticum, an antibioticum, an anti-infective agent, antidepressivum and/or antipsychoticum, an antidiabeticum, an antidot, an antiemeticum, antiepilepticum, antifibrinolyticum, anticonvulsivum or anticholinergicum, an enzyme, a coenzyme or the corresponding enzyme inhibitor, an antihistaminicum (and combinations thereof) or antihypertonicum, an antihypotonicum, anticoagulant, antimycoticum, antimyasthenicum, an agent against Morbus Alzheimer or Morbus Parkinson, an agent for ACS therapy, an antiphlogisticum, antipyreticum, antirheumaticum, antisepticum, a respiratory analepticum or a respiratory stimulant, a broncholyticum, cardi tonicum, chemotherapeuticum, a coronary dilatator, a cytostaticum, a diureticum, a ganglium-blocker, a glucocorticoid, an anti-flew agent, a haemostaticum, hypnoticum, an immunoglobuline or its fragment or any other immunologically active substance, such as an immunomodulator, a bioactive carbohydrate (derivative), a contraceptive, an anti-migraine agent, a corticosteroid, a muscle relaxant, a narcoticum, a neurotherapeuticum, a (poly)nucleotide, a neurolepticum, a neurotransmitter, a (poly)peptide (derivative), an opiate, an opthalmicum, (para)-sympaticomimeticum or (para)sympathicolyticum, a protein(derivative), a psoriasis/neurodermitis drug, a mydriaticum, a psychostimulant, rhinologicum, a sleep-inducing agent, a sedating agent, a spasmolyticum, tuberculostaticum, urologicum, a vasoconstrictor or vasodilatator, a virustaticum, a wound-healing substance, an alcohol abuse preparation, an anticonvulsant, an antineoplastic, an antirheumatic, an appetite suppressant, a biological response modifier, a blood modifier, a bone metabolism regulator, a cardioprotective agent, a cardiovascular agent, a central nervous system stimulant, an enzyme, an agent for erectile dysfunction therapy, a fertility agent, a gastrointestinal agent, a gout preparation, a hormone, an agent for hypercalcemia management, an agent for hypocalcemia management,

an immunosuppressive, a migraine preparation, a motion sickness product, an agent for multiple sclerosis management, a muscle relaxant, a nutritional, an ophthalmic preparation, an osteoporosis preparation, an otic preparation, a parasympatholytic, a parasympathomimetic, a prostaglandin, a psychotherapeutic agent, a respiratory agent, a sedative & hyponotic, a skin & mucous membrane agent, a smoking cessation aid, a sympatholytic, a tremor preparation, a urinary tract agent, a vaginal preparation, a vertigo agent, an immunologically active substance (such as an immunomodulator, e.g., bacterial extracts or cell wall components like cholera toxin, heat labile toxin, monophosphoryllipid A, or cytokine inducing agents or hormones like thymosin, thymulin, thymopoietin, or phytoimmunostimulants like extracts from Echinacea root, wild indigo root, white cedar leave tips, or synthetic immunomodulators like quinoline derivatives, synthetic peptides, pyrimidine, lipopeptides, or cytokines or immunosuppressants, and signal transduction inhibitors like cyclosporin A, FK506, FTY720, rapamycin), an inhibitor (antagonist), or a promotor (agonist) of the activity of any of above mentioned agents, or any combination of said active substances.

6. The use of claim 3 or the pharmaceutical composition of claim 4 wherein the antigen is derived from a pathogen.
7. The use of claim 3 or the pharmaceutical composition of claim 4 wherein said pathogen belongs to extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus*, gram-negative bacteria, such as *Meningococcus* and *Gonococcus* species, species of *Neisseria*, gram negative bacteria, including enteric organisms such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Diphtheria*, *Bordetella Pertussis*, and gram-positive bacteria (e.g. *Bacillus pestis*, *BCG*), particularly anaerobes, such as the *Clostridium* species (e.g. *Clostridium tetani*, *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*), bacteria and viruses, which survive and replicate within host cells, comprising mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes*, retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths,

- and ectoparasites, such as ticks and mites, or *Brucella* species, (e.g. *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae*, *B. ovis*, the causative agent for cholera (e.g. *Vibrio cholerae*), *Haemophilus* species like *H. actinomycentemcomitans*, *H. pleuropneumoniae* , as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases or to eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body which do not necessarily result from microbial infections.
8. The use of claim 3 or the pharmaceutical composition of claim 4 wherein the antigen is used in a purified or even better in a pure form.
 9. The use of claim 3 or the pharmaceutical composition of claim 4 wherein the antigen is the antigenic determinant of hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, *Haemophilus* species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases or else eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body, which do not necessarily result from microbial infections.
 10. The use of claim 3 or the pharmaceutical composition of claim 4, wherein the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.
 11. The use of claim 3 or the pharmaceutical composition of claim 4 wherein the allergen belongs to the class of the inhalation allergens, including but not limited to various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; furthermore, food and drug allergens; contact allergens; injection, invasion or depot allergens, such as

various (gastrointestine-resident) worms, echinococci, trichines, etc., a part of implantation material.

12. The use of any one of claims 1 to 3 and 5 to 11 or the pharmaceutical composition of any one of claims 4 to 9 additionally comprising a compound which releases or induces cytokine or anti-cytokine activity or exerts such an activity itself.
13. The use or the pharmaceutical composition of claim 12 wherein the compound exerting cytokine activity is IL-4, IL-3, IL-2, TGF, IL-6, TNF, IL-1 α and/or IL-1 β , a type I interferon, preferably IFN-alpha or IFN- β , IL-12, IFN- γ , TNF- β , IL-5 or IL-10.
14. The use or the pharmaceutical composition of claim 12 wherein said compound with anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative, or an analogue thereof.
15. The use or the pharmaceutical composition of claim 4 wherein the compound displaying or inducing cytokine or anti-cytokine activity and the pharmaceutically active ingredient or antigen or allergen are associated with the penetrant.
16. The use of any one of claims 1 to 15 or the pharmaceutical composition of any one of claims 4 to 15 wherein the less soluble self-aggregating molecule is a lipid, preferably a polar lipid, and the more soluble component is a surfactant or some more soluble form of the polar/basic lipid.
17. The use of any one of claims 1 to 16 or the pharmaceutical composition of any one of claims 4 to 16 wherein the more soluble component is an agent to be transported across the barrier, said agent having a tendency to form common large structures with the less soluble component(s) of the penetrant, typically in the form of a physical or a chemical complex.
18. The use of any one of claims 1 to 17 or the pharmaceutical composition of any one of claims 4 to 17 wherein the more soluble component tends to solubilise the penetrating droplet and is present in concentration not exceeding 99 mol%

of the concentration required to disintegrate the droplet or, alternatively, not exceeding 99 mol% of the saturating concentration in the unsolubilised droplet, whichever is higher, values below 50% of the former relative concentration being particularly useful, with values below 40 rel-% or even around and below 30 rel-% being even more advantageous, whereas in the case of droplets which cannot be solubilised by the more soluble component relative concentrations which exceed the above mentioned relative concentrations by the factor of up to 2 are most preferred.

19. The use of any one of claims 1 to 18 or the pharmaceutical composition of any one of claims 4 to 18 wherein the less soluble penetrant component is a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of a lipid, preferably a polar lipid which is sufficiently soluble for the purpose of this invention.
20. The use of any one of claims 1 to 19 or the pharmaceutical composition of any one of claims 4 to 19 wherein the average penetrant diameter is between 25 nm and 500 nm, preferably between 30 nm and 250 nm, even more preferably between 35 nm and 200 nm and particularly preferably between 40 nm and 150 nm.
21. The use of any one of claims 1 to 20 or the pharmaceutical composition of any one of claims 4 to 20 wherein the penetrant concentration in the formulation for the use in human or animal nose is 0.001 to 20 weight-% of total dry mass in the formulation, in particular between 0.01 w-% and 15 w-%, more preferably between 0.1 w-% and 12.5 w-% and most preferred between 0.5 w-% and 10 w-%.
22. The use of any one of claims 1 to 21 or the pharmaceutical composition of any one of claims 4 to 21 wherein the supporting medium, e.g. a buffer, is selected to be a biocompatible solution with an osmotic activity similar to that of a monovalent electrolyte with concentration in the range between 1 mM and 500 mM, more preferably between 10 mM and 400 mM, even more preferably between 50 mM and 300 mM, and most preferably between 100 mM and 200

mM or else such solution that affords practically sufficient penetrant stability combined with practically sufficient transport rate across the barrier.

23. The use of any one of claims 1 to 22 or the pharmaceutical composition of any one of claims 4 to 22 wherein the relative drug or agent concentration is between 0.001 and 40 weight-% of total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 25 w-% and most preferably between 0.5 w-% and 15 w-%.
24. The use of any one of claims 1 to 23 or the pharmaceutical composition of any one of claims 4 to 23 wherein the medium supporting the drugs and carriers is a biocompatible buffer with pH value between 4 and 10, more frequently between 5 and 9 and most often between 6 and 8.
25. The use of any one of claims 1 to 24 or the pharmaceutical composition of any one of claims 4 to 24 wherein the additives are included in the preparation to reduce the system sensitivity to chemical, biological or ambient stress, including anti-oxidants, antagonists of undesired enzyme action, cryo-preservants, microbicides, etc., or else modulators of physically important system properties, such as formulation viscosity, etc..
26. The use of any one of claims 1 to 25 or the pharmaceutical composition of any one of claims 4 to 25 wherein the relative drug or agent dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.1x and 500x, more often between 0.5x and 250x, and even more preferably between 1x and 100x different from the corresponding drug or agent dose that would have to be injected to achieve the desired biological effects.
27. The use of any one of claims 1 to 26 or the pharmaceutical composition of any one of claims 4 to 26 wherein the applied penetrant dose is between 0.01 mg and 15 mg per nostril, even more often is in the range 0.1 mg and 10 mg per nostril, and preferably is between 0.5 mg and 5 mg per nostril.

28. The use of any one of claims 1 to 27 or the pharmaceutical composition of any one of claims 4 to 27 wherein the efficiency of administration and the biological effects of the agent or drug chosen are controlled by using different application volumes.
29. The use of any one of claims 1 to 28 or the pharmaceutical composition of any one of claims 4 to 28 wherein said formulation is administered using a metered delivery device.
30. The use of any one of claims 1 to 29 or the pharmaceutical composition of any one of claims 4 to 29 wherein different application volumes are selected to control the efficiency of administration and the biological effects of the chosen agent or drug.
31. The use of any one of claims 1 to 30 or the pharmaceutical composition of any one of claims 4 to 30 wherein the penetrants in suspension are loaded with the drugs or agents within 24 hours prior to the formulation administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the resulting formulation administration in the nose.
32. The use of any one of claims 1 to 31 or the pharmaceutical composition of any one of claims 4 to 31 wherein the delivery device is loaded at the treatment site.
33. The use of any one of claims 1 to 32 or the pharmaceutical composition of any one of claims 4 to 32 wherein the device is loaded separately with penetrants and the molecules, particularly biological agents, to be associated therewith.
34. The use of any one of claims 1 to 33 or the pharmaceutical composition of any one of claims 1 to 33 wherein the pharmaceutically active ingredient is for administration to the nervous system.
35. The use or the pharmaceutical composition of claim 34 wherein the nervous system is the brain.

36. The use of any one of claims 1 to 35 or the pharmaceutical composition according to any one of claims 4 to 35 wherein said pharmaceutical composition is a vaccine.
37. The vaccine of claim 36 which further comprises a pathogen extract or a compound from a pathogen or a fragment or a derivative thereof.
38. The vaccine of claim 37 wherein said pathogen extract or compound is selected from hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps or polio viruses, cytomegalovirus, rhinovirus, etc., or fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, *Haemophilus* species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus or rubella diseases.
39. The vaccine of any one of claims 36 to 38 which further comprises an adjuvant.
40. The vaccine of claim 38 or 39 wherein said adjuvant is lipopolysaccharide, such as lipid A or a derivative or modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a microorganism; an extract of a microorganism, including bacterial exo- and endotoxins, preferably cholera toxin or the heat labile toxin of *E. coli*, an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, an LT halotoxin, purified protein derivative of *M. tuberculosis*, LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes*, or outer membrane protein of group B *Neisseria meningitidis* (GBOMP), bacterial or viral nucleic acids, such as oligonucleotides comprising unmethylated CpG dinucleotides.
41. The vaccine of any one of claims 36 to 40 comprising a blend of MPL and IL-12 or GM-CSF and IL-4.

42. The vaccine of any one of claims 36 to 41 wherein the relative immunogen/antigen dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.01x and 100x, more often between 0.05x and 75x, and even more preferably between 0.1x and 50x different from the corresponding immunogen/antigen dose that would have to be injected to achieve the desired biological effect.
43. The vaccine according to any one of claims 39 to 42 wherein the concentration of the transnasally administered adjuvant is between 10x lower and up to 1000x higher than that used with the corresponding subcutaneously injected formulations employing similar antigen, the transnasally administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.
44. A container comprising the pharmaceutical composition according to any one of claims 4 to 43.
45. A package comprising at least one container comprising the pharmaceutical composition of any one of claims 4 to 43.
46. A method for generating a protective immuno response on a mammal by vaccinating said mammal with a vaccine according to any one of claims 36 to 43.
47. The method according to claim 46 wherein different administration volumes are selected to control the applied immunogen dose and the outcome of vaccination.
48. The method according to claim 46 or 47, wherein a suspension of antigen-free penetrants is loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before administering the resulting formulation in the nose.

49. The method of any one of claims 46 to 48 characterised in that at least one dose of vaccine is administered.
50. The method according to claim 49 wherein said vaccine is administered as a booster vaccination.
51. The method according to any one of claims 46 to 50, wherein the vaccine is applied between 2 and 10, preferably between 2 and 7, even more preferably up to 5 and most preferably up to 3 times, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined according to a suitable assessment method, or else to deem the effort as having failed.
52. The method according to any one of claims 48 to 51, wherein the time interval between the subsequent vaccinations is chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years.
53. The method according to any one of claims 46 to 52, wherein the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is determined as a function of a suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further.

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Intranasal application of Transfersulin
IDDM test person, ~0.8 IU/kg

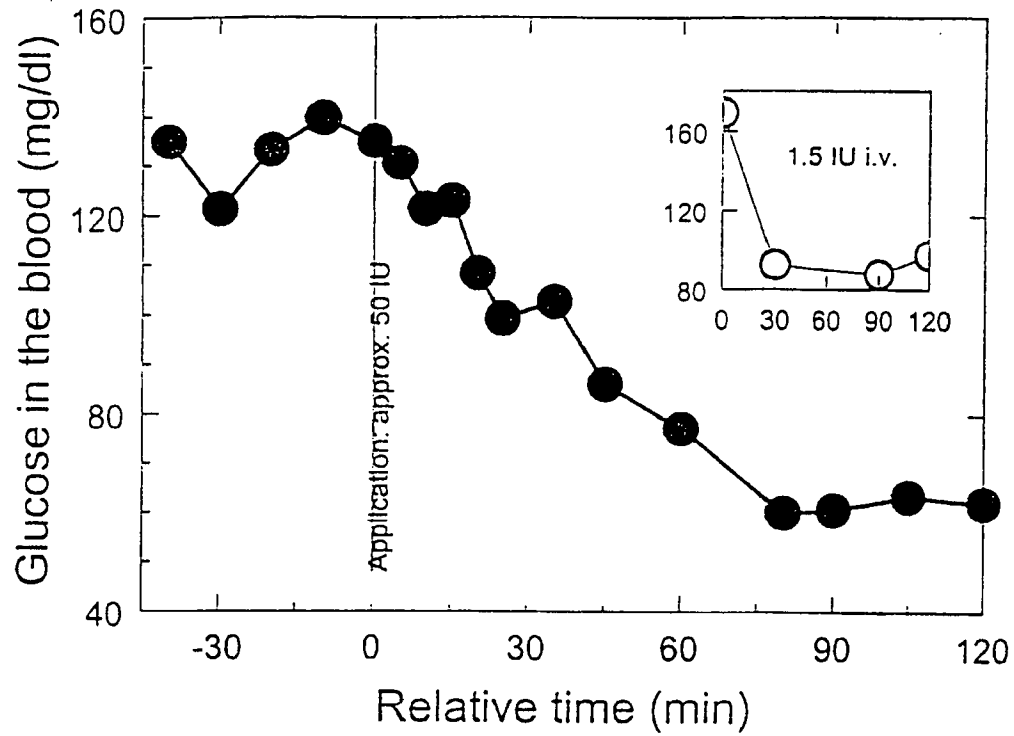


Fig. 1

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Intranasal application of Transfersulin
on a normoglycaemic test person, - 0.15 IU/kg (2x)

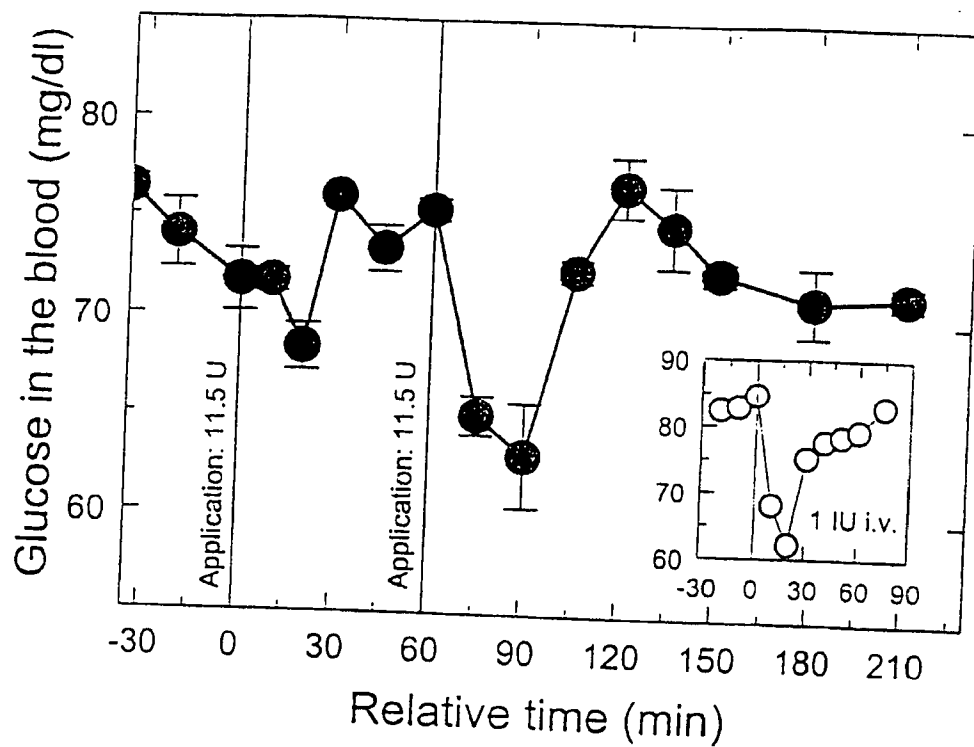


Fig. 2

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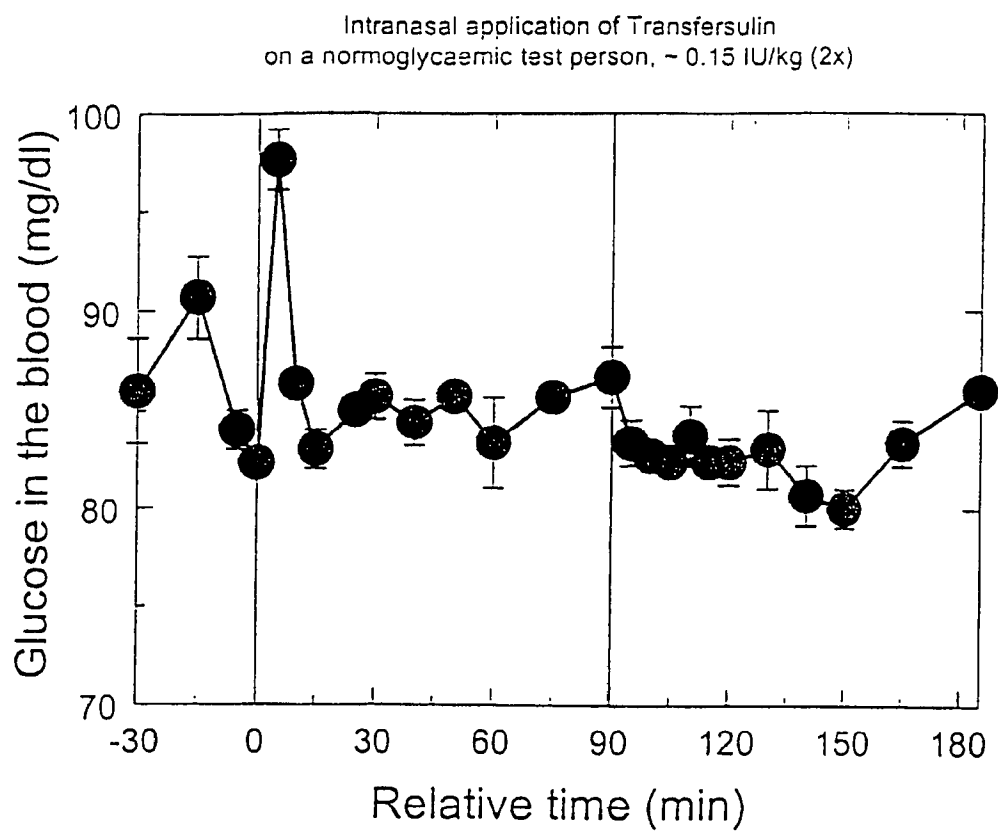


Fig. 3a

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Intranasal application of Transfersulin
in IDDM test person, ~0.4 IU/kg

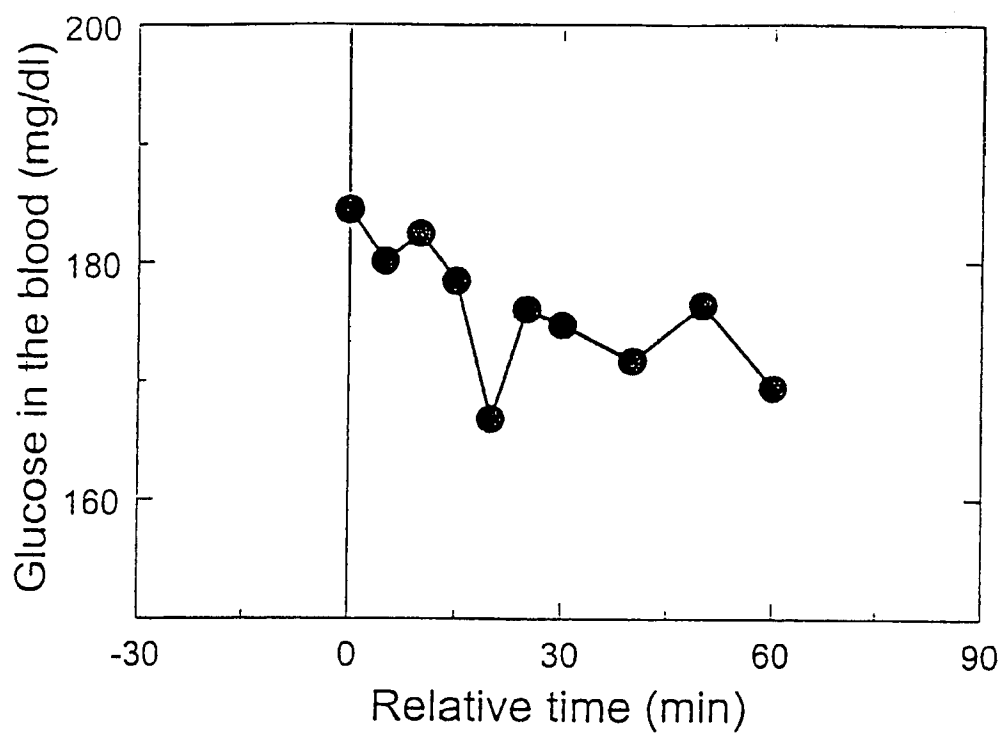


Fig. 3b

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Effects of nasally administered
cytokines on specific immune response,
1st boost + 7 d

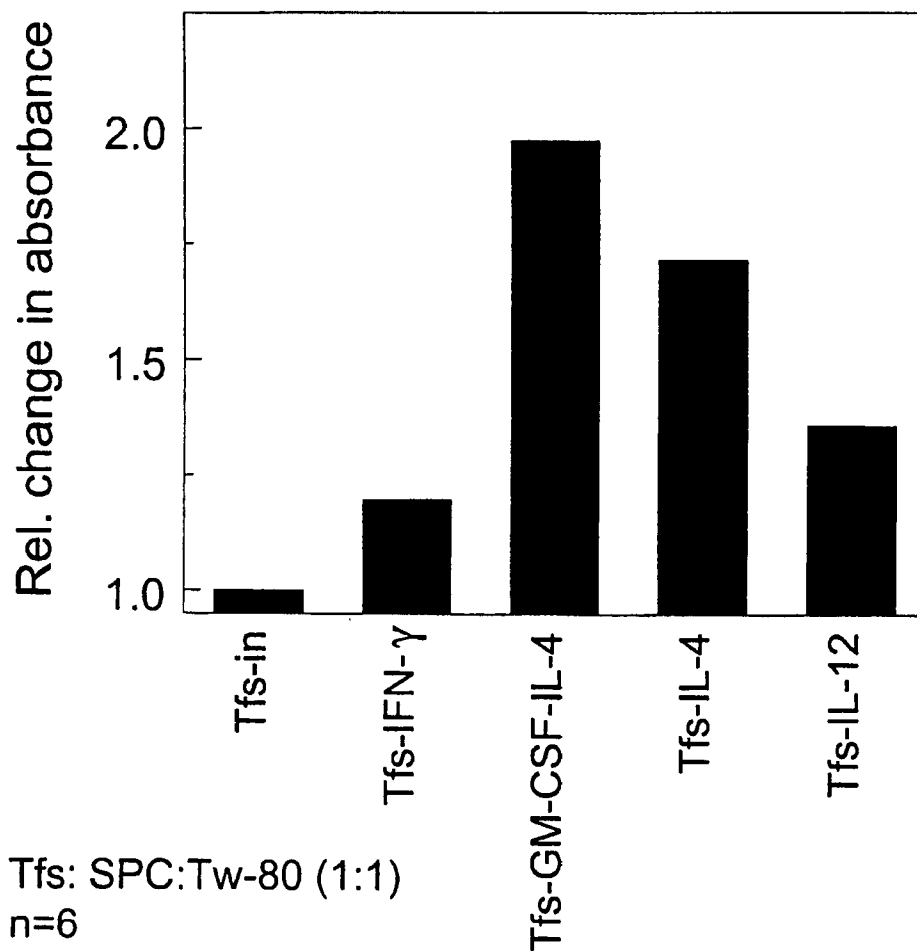


Fig. 4

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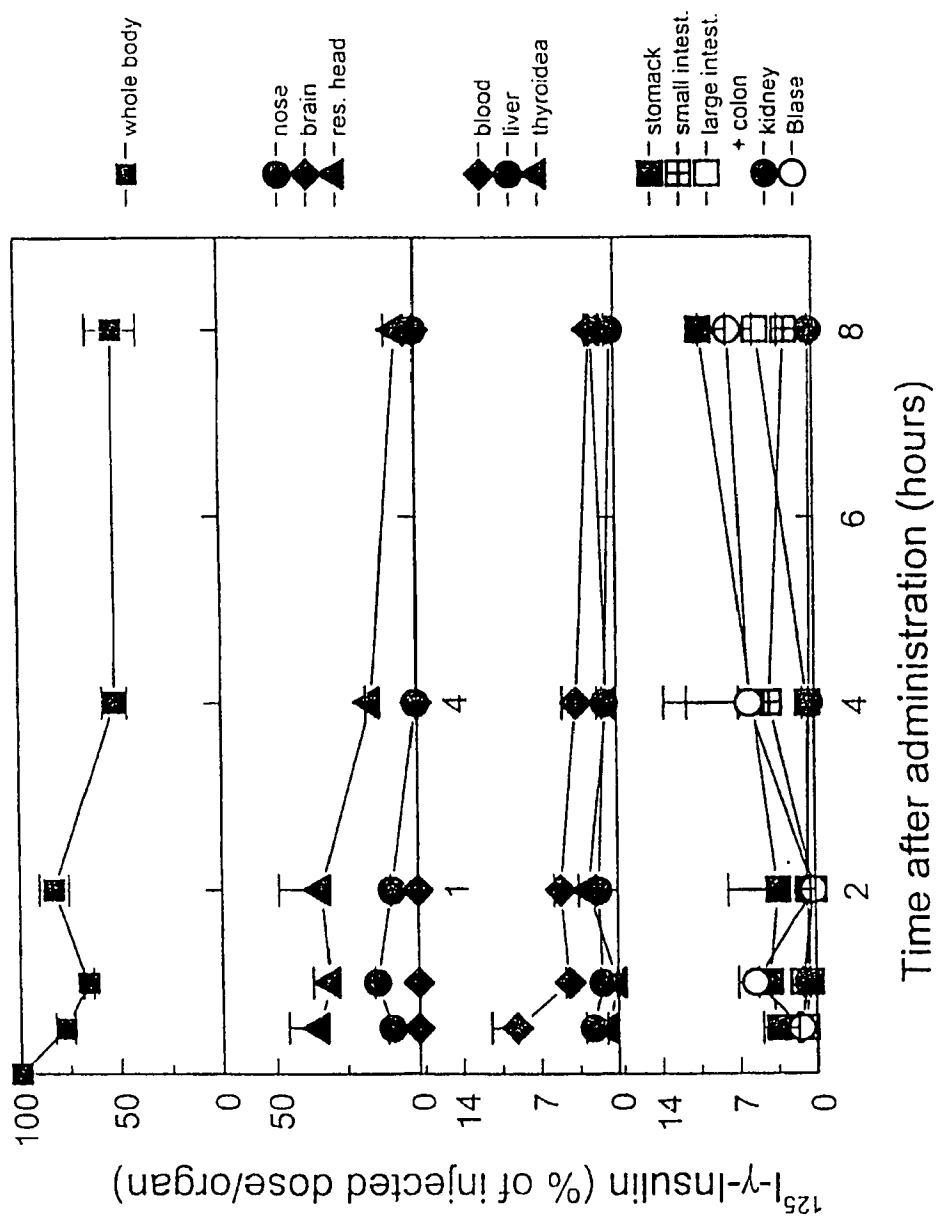


Fig. 5

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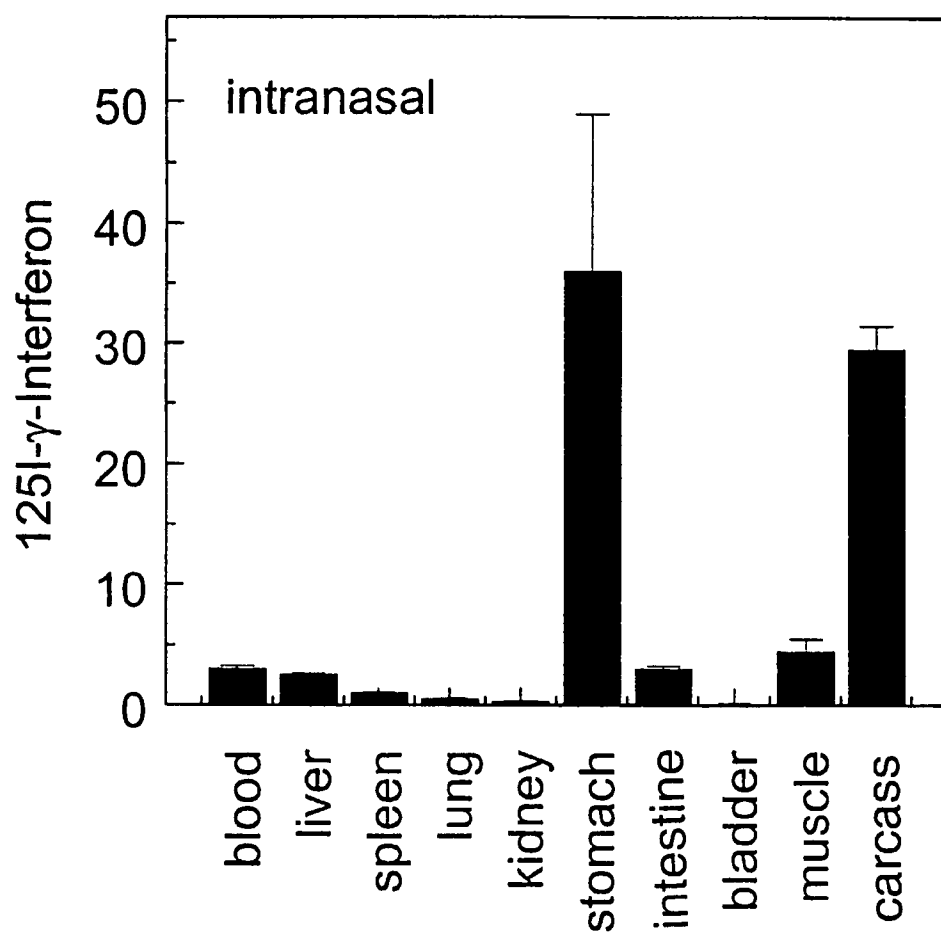


Fig. 6

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Intranasal administration: Tetanus toxoid (TT, impure)

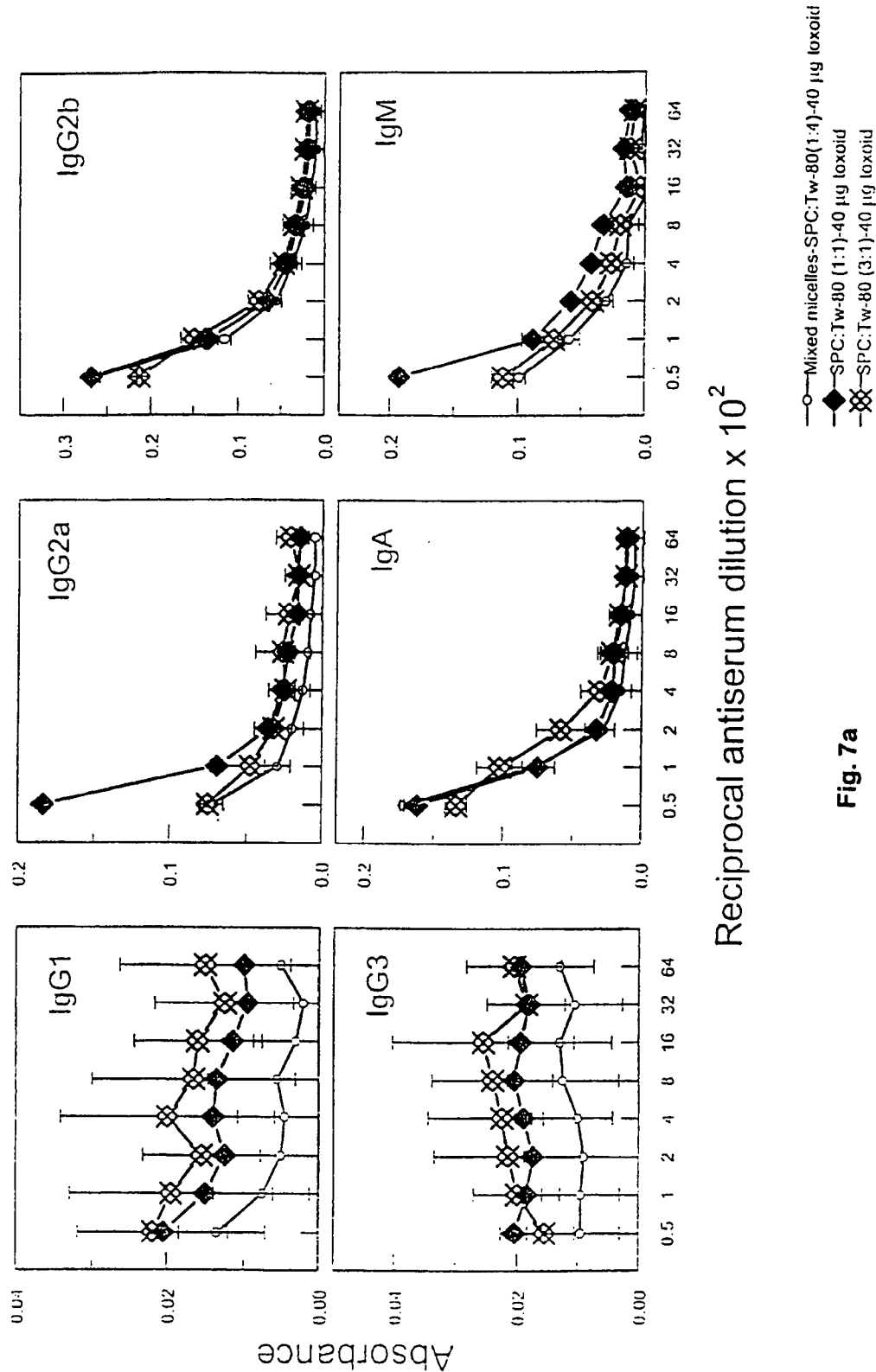


Fig. 7a

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Intranasal immunisation: Tetanus toxoid (TT impure)

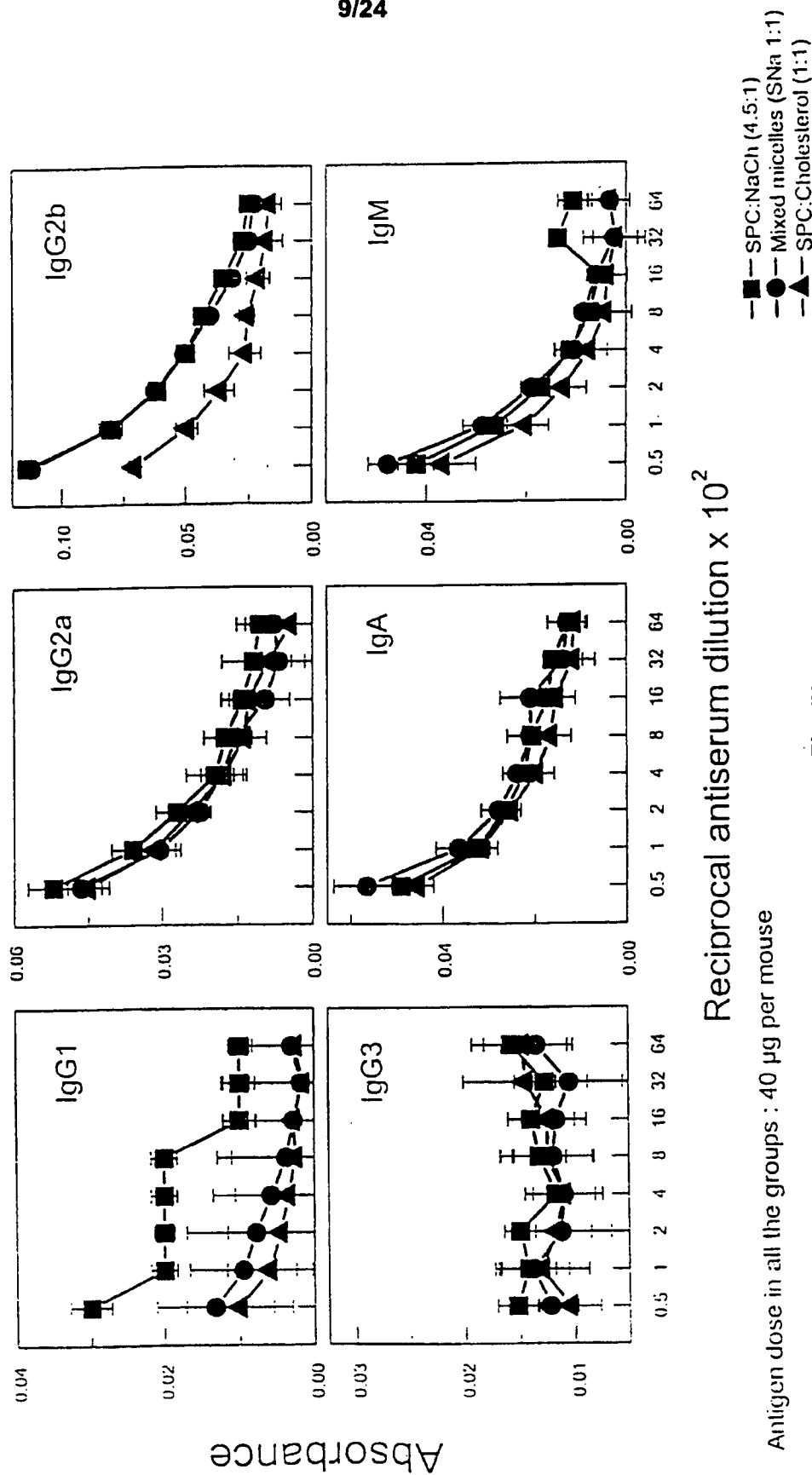


Fig. 7b

Reciprocal antiserum dilution x 10²

Antigen dose in all the groups : 40 µg per mouse

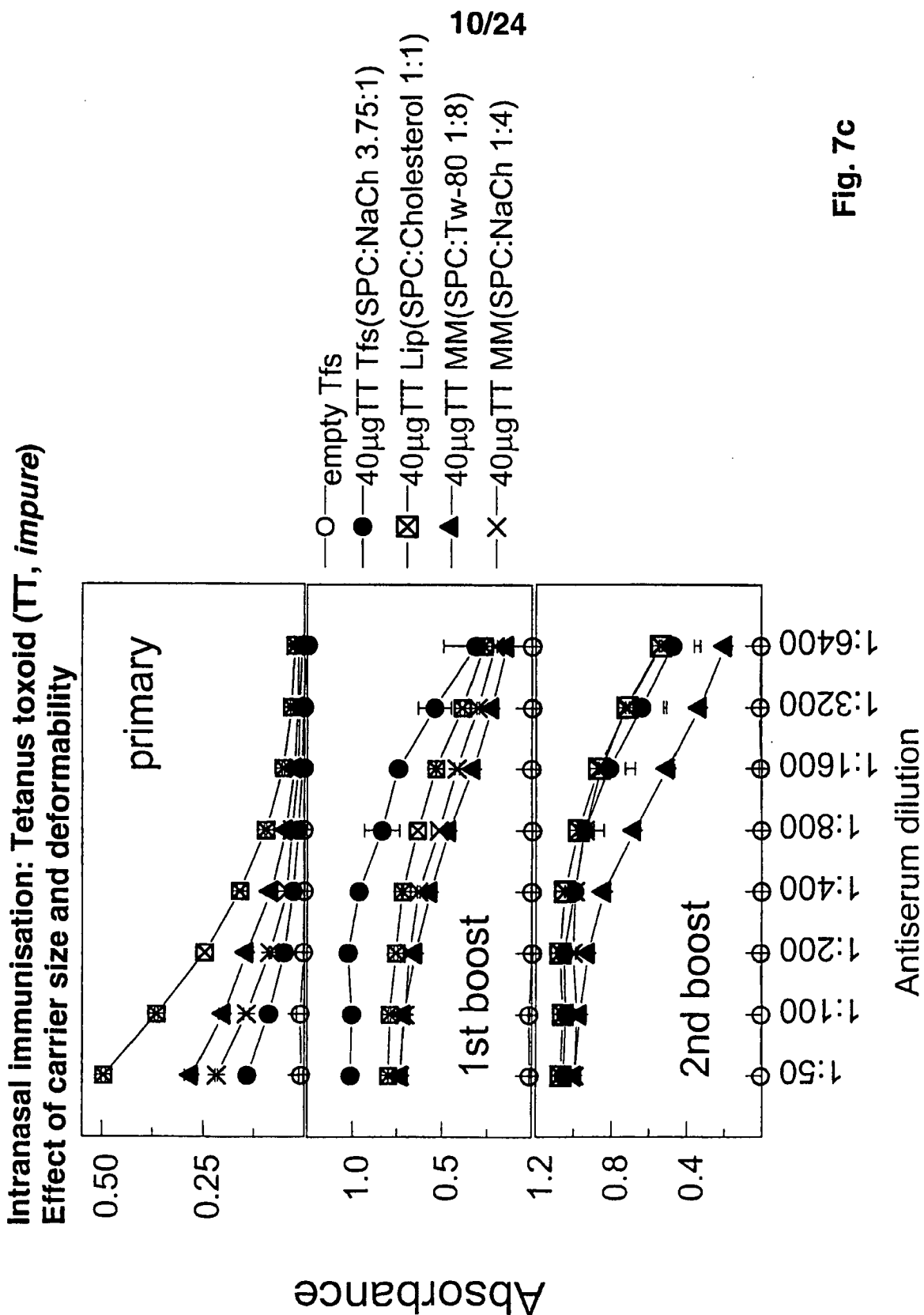


Fig. 7c

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Intranasal Immunization: Tetanus toxoid, (TT, purified)
dose effect

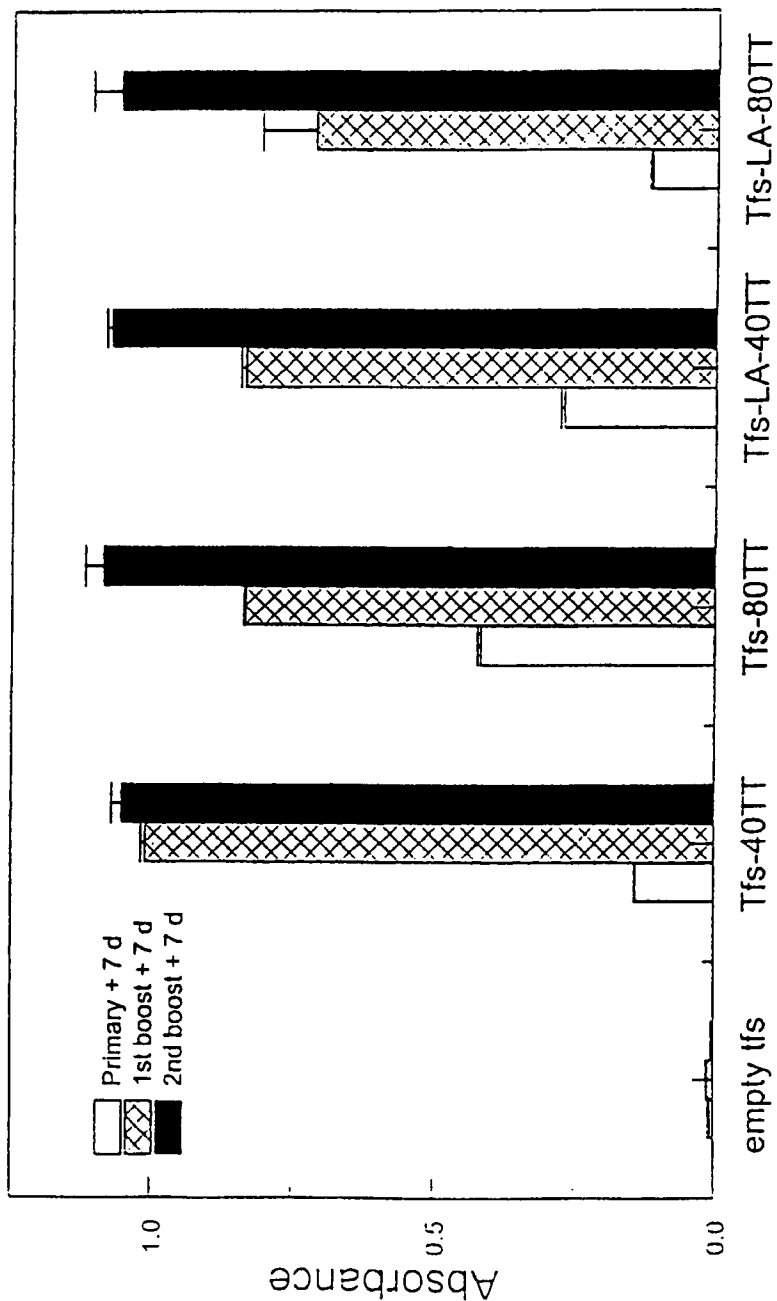


Fig. 8a

Intranasal immunisation: Tetanus toxoid (TT, purified), Effect of antigen dose

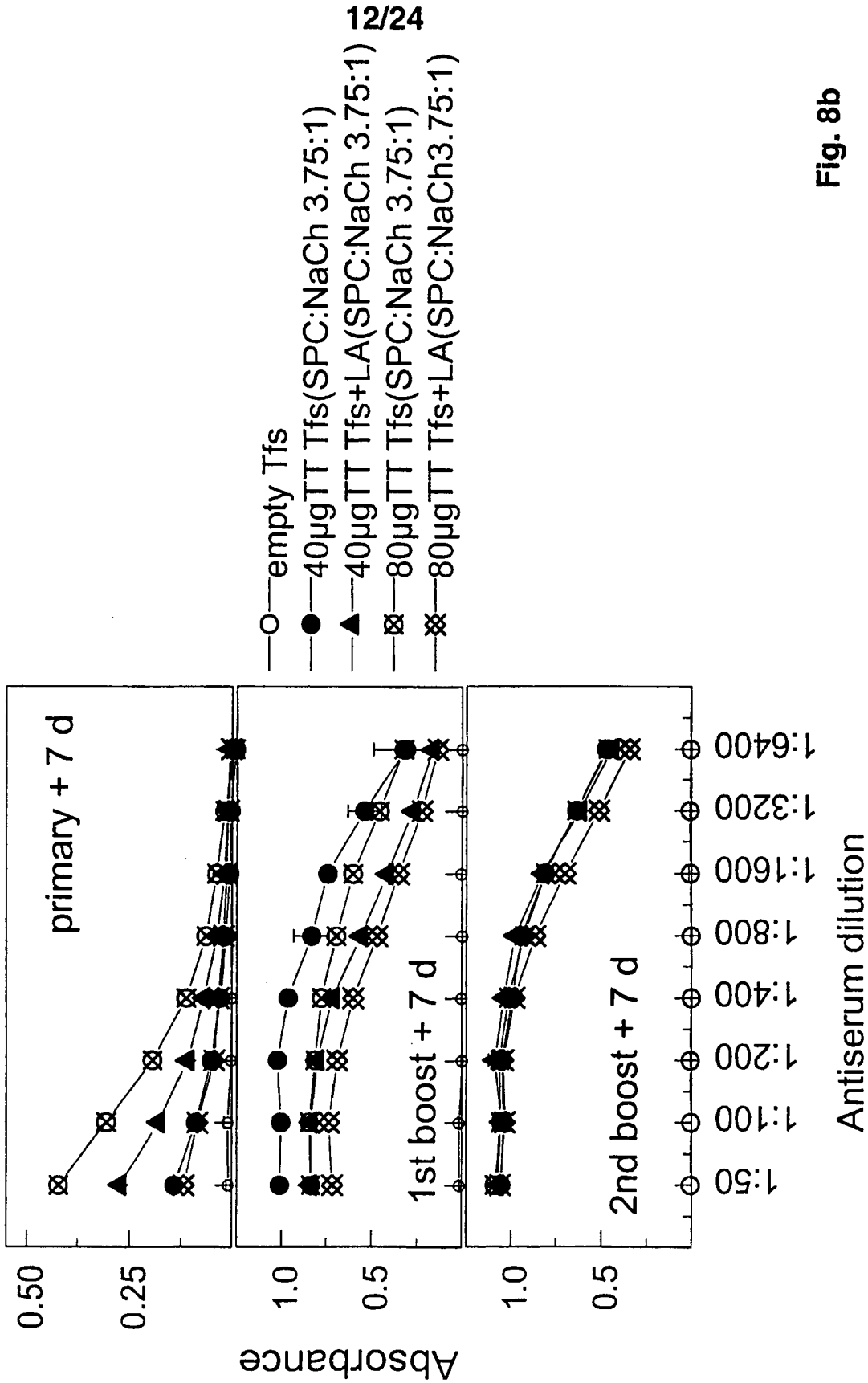
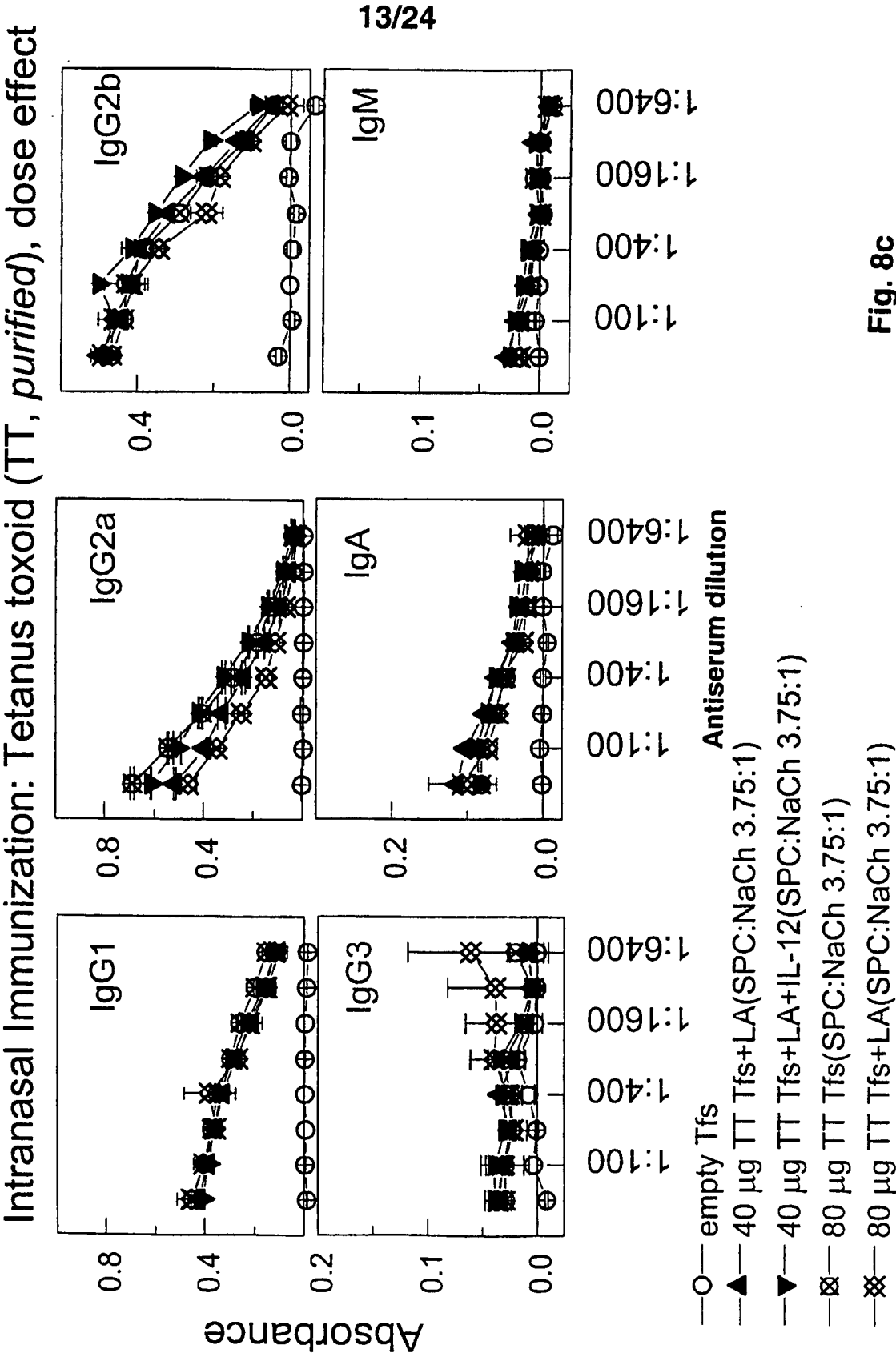


Fig. 8b



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Noninvasive immunisation: Tetanus toxoid (TT, purified)
effect of application route

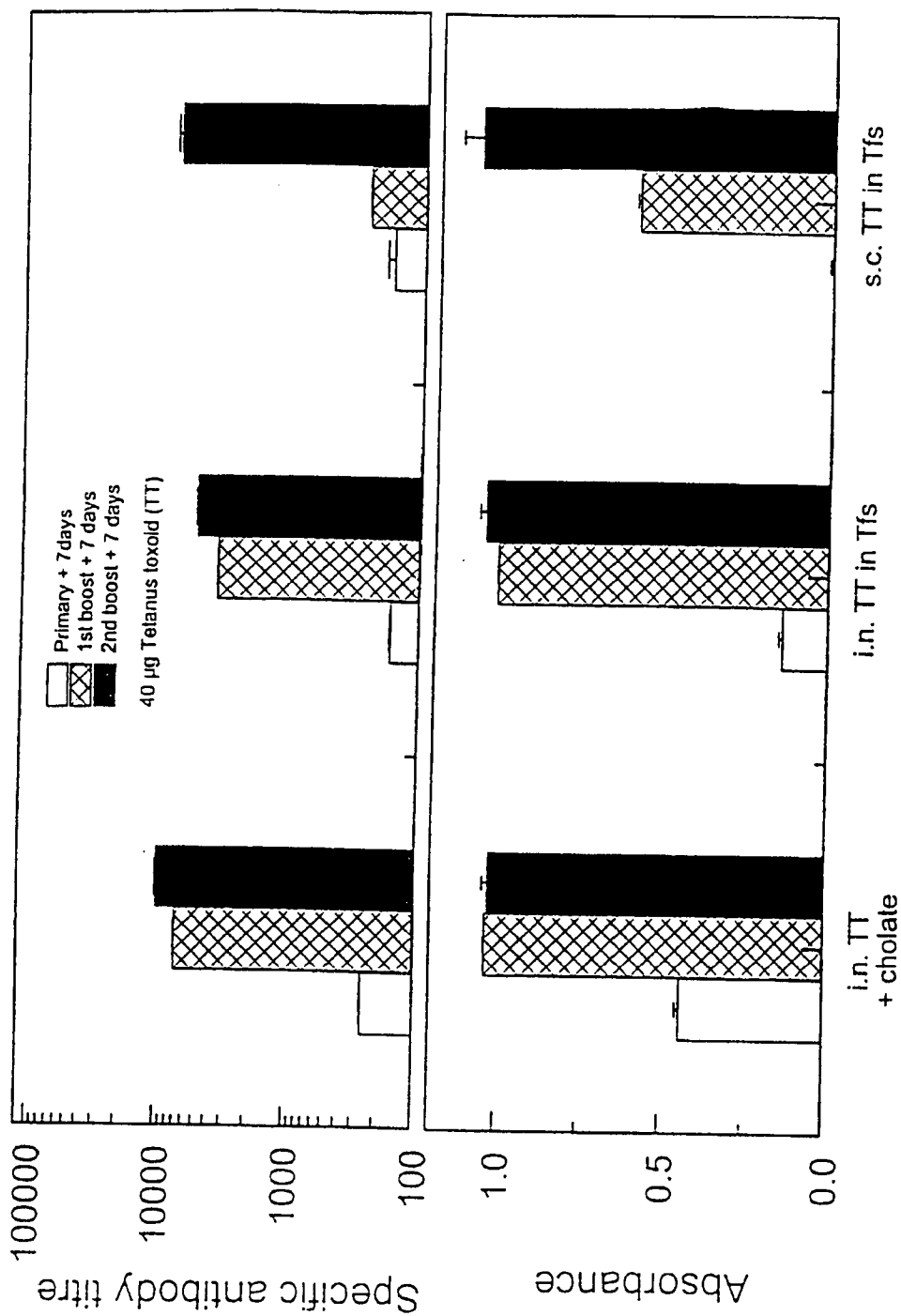


Fig. 9a

Noninvasive immunisation: Tetanus toxoid (TT, *purified*) route of application effect

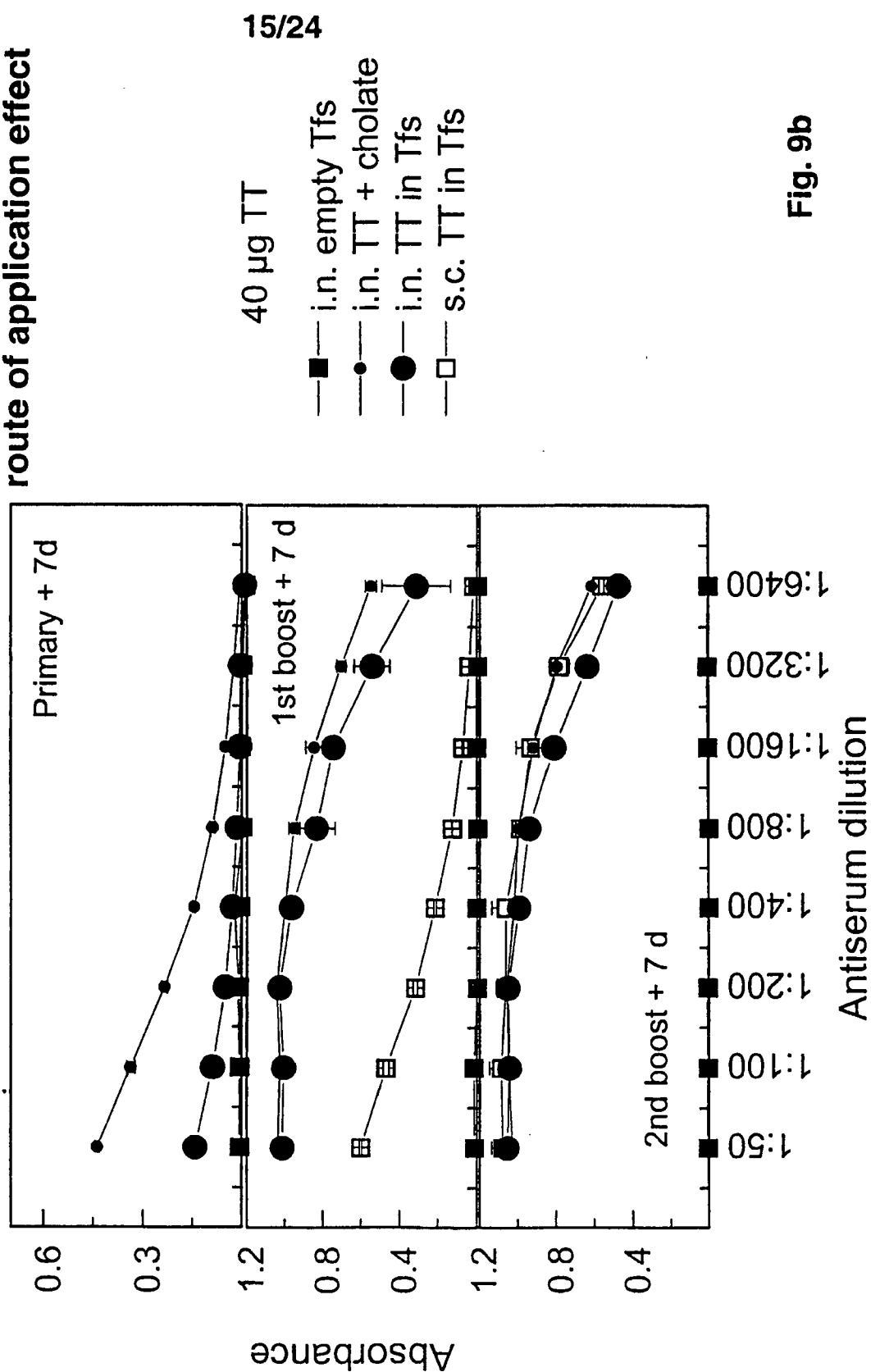
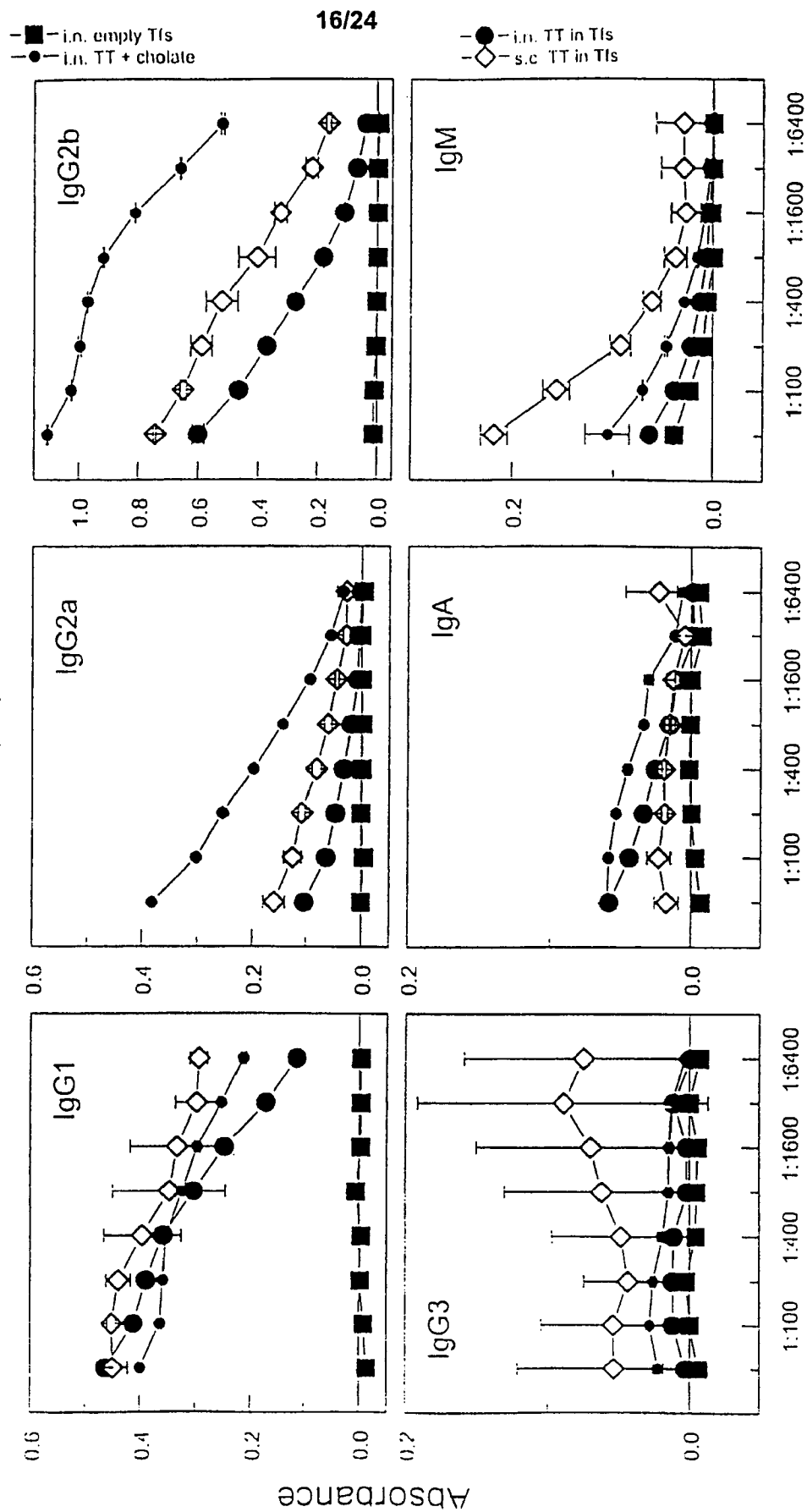


Fig. 9b

Antigen-specific Isotyping: Intranasal / Subcutaneous

40 µg Tetanus Toxoid (TT), ultrafiltered



Antiserum dilution

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Intranasal immunisation: Tetanus toxoid (TT)

dose and route of administration effect

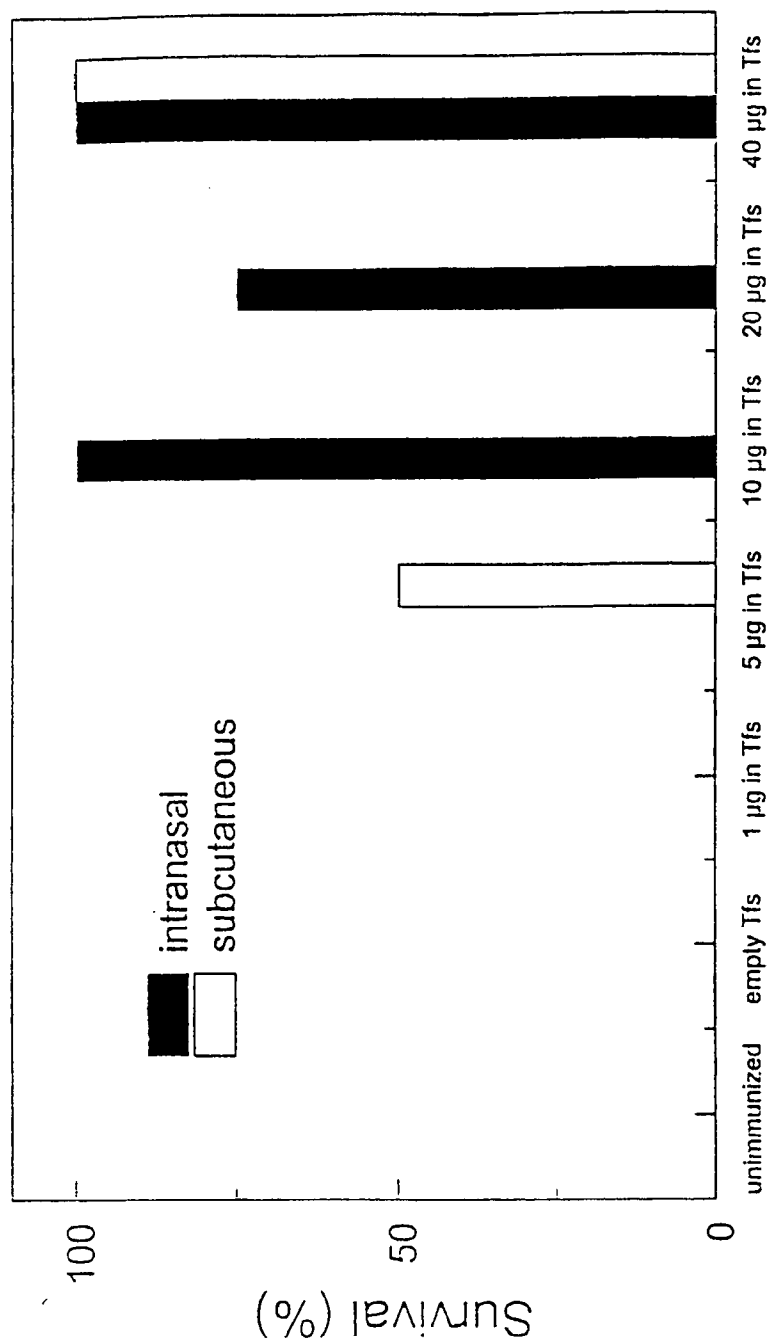


Fig. 10a

Intranasal immunisation: Tetanus toxoid (TT, purified) dose effect

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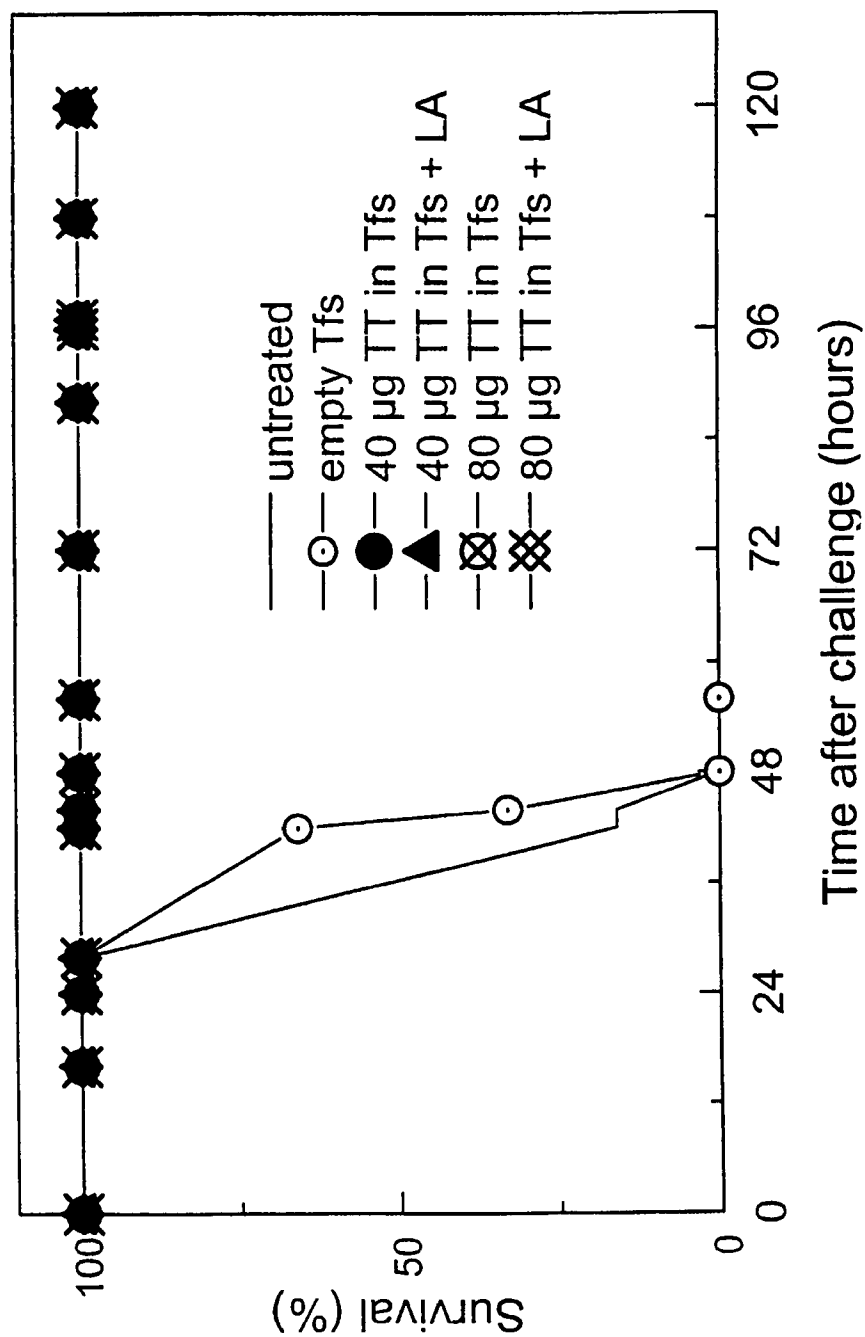


Fig. 10b

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Intranasal immunisation: Tetanus toxoid (TT, *impure*)
effect of immunomodulators

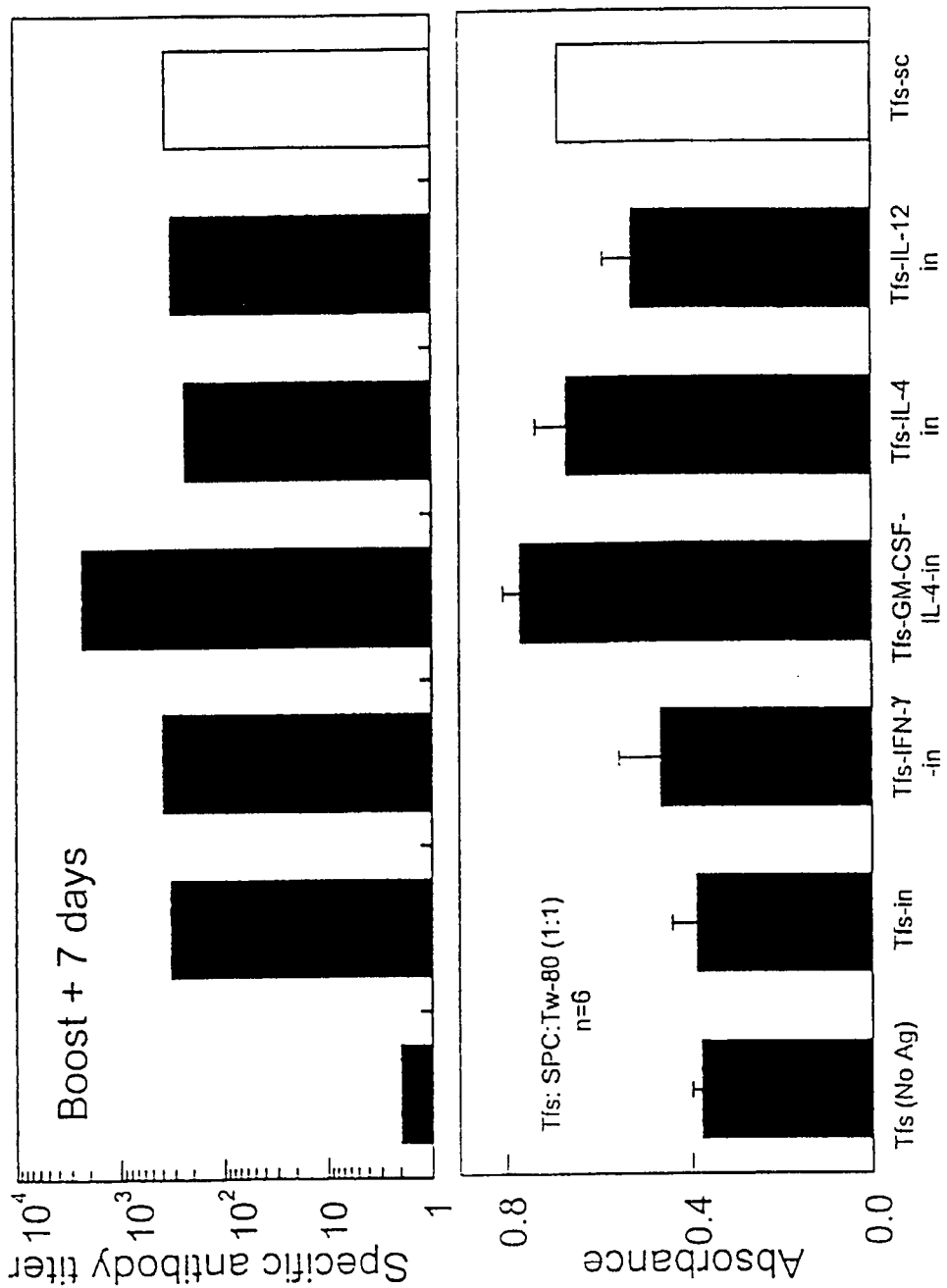


Fig. 11a

Intranasal immunisation: Tetanus toxoid (TT, impure, 40 µg), effect of immunomodulators,

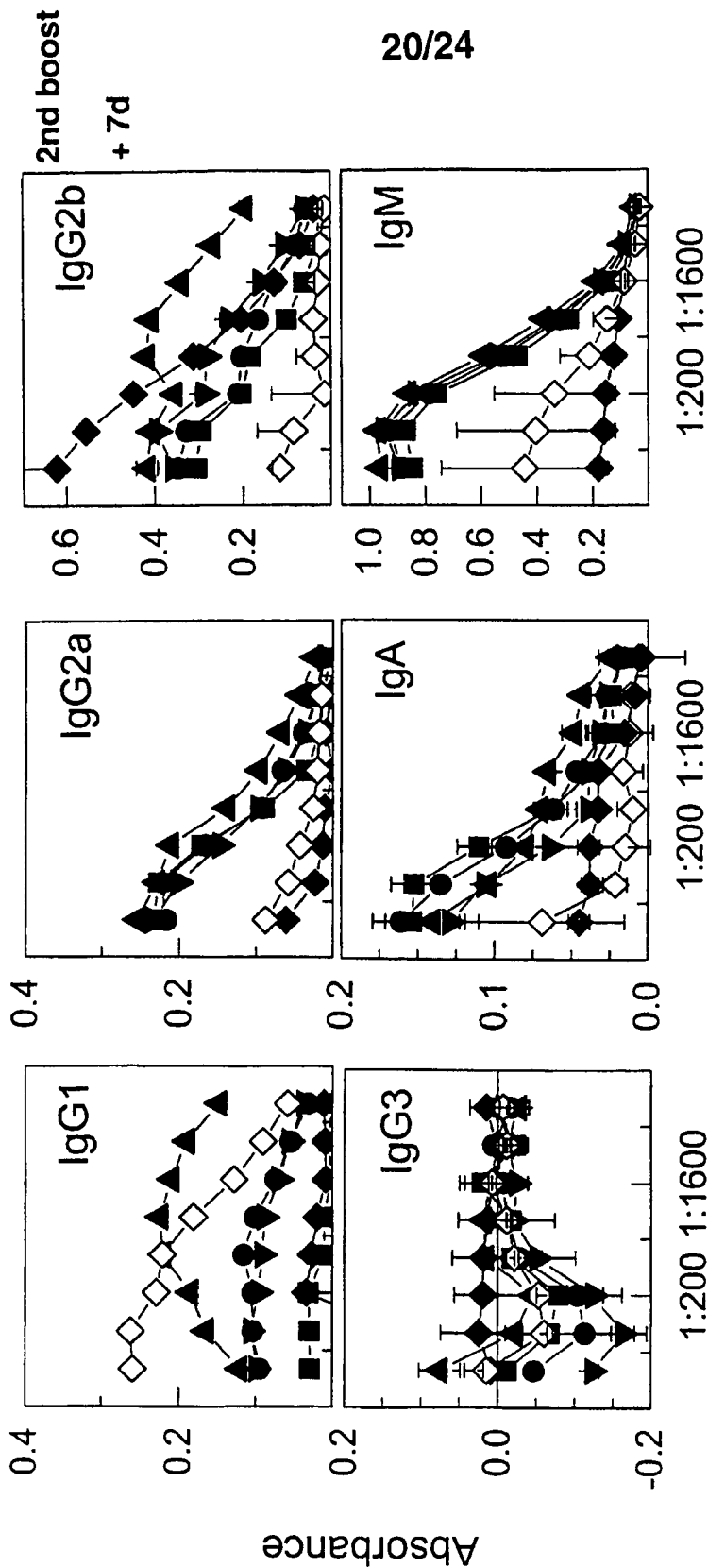


Fig. 11b

SPC:Tw-80 3.5:1

- ◇ i.n. TT in Tfs
- i.n. TT in Tfs + IFN-γ
- ▲ i.n. TT in Tfs + GM-CSF + IL-4
- ▼ i.n. TT in Tfs + IL-4
- ◆ i.n. TT in Tfs + IL-12
- s.c. TT in Tfs

Intranasal immunisation: Tetanus toxoid (TT, purified)
effect of immunopotentiators

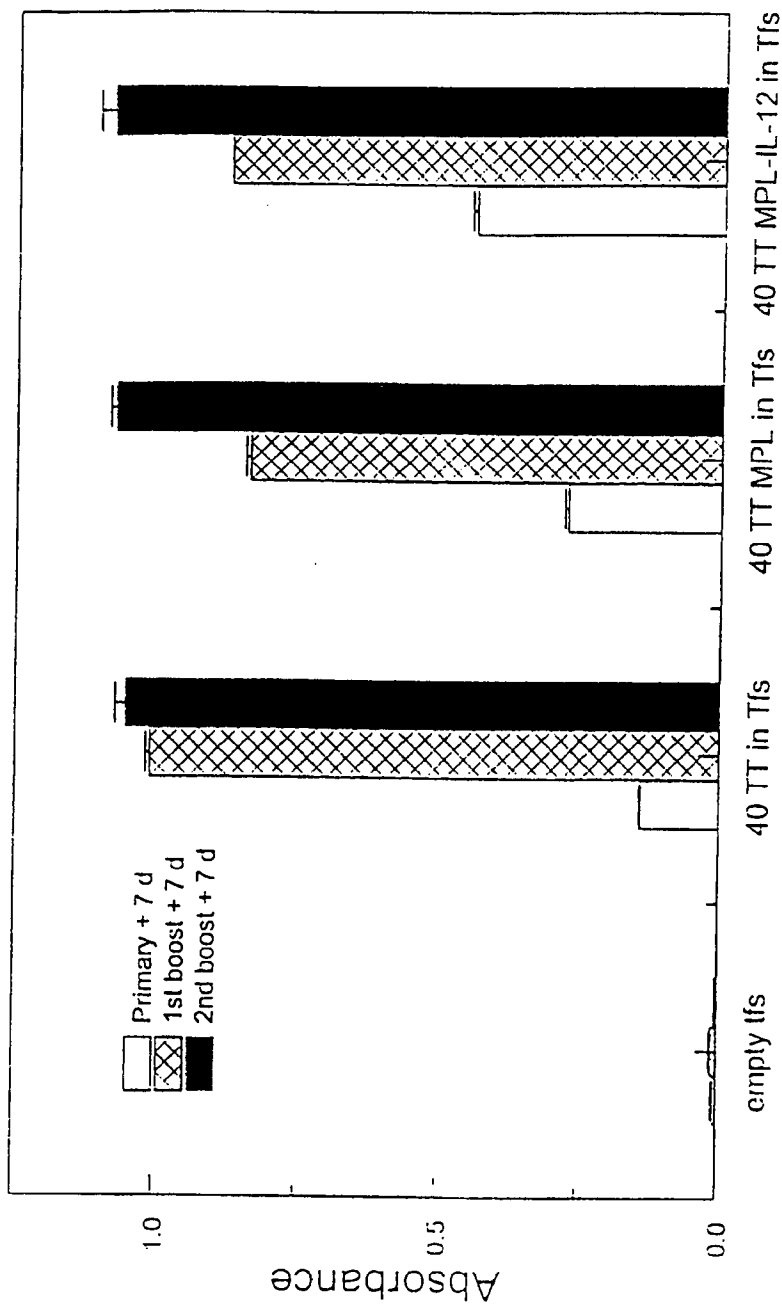


Fig. 12

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Potential of nasal immune response to tetanus
toxoid (TT) by bacterial adjuvant, cholera toxin (CT)

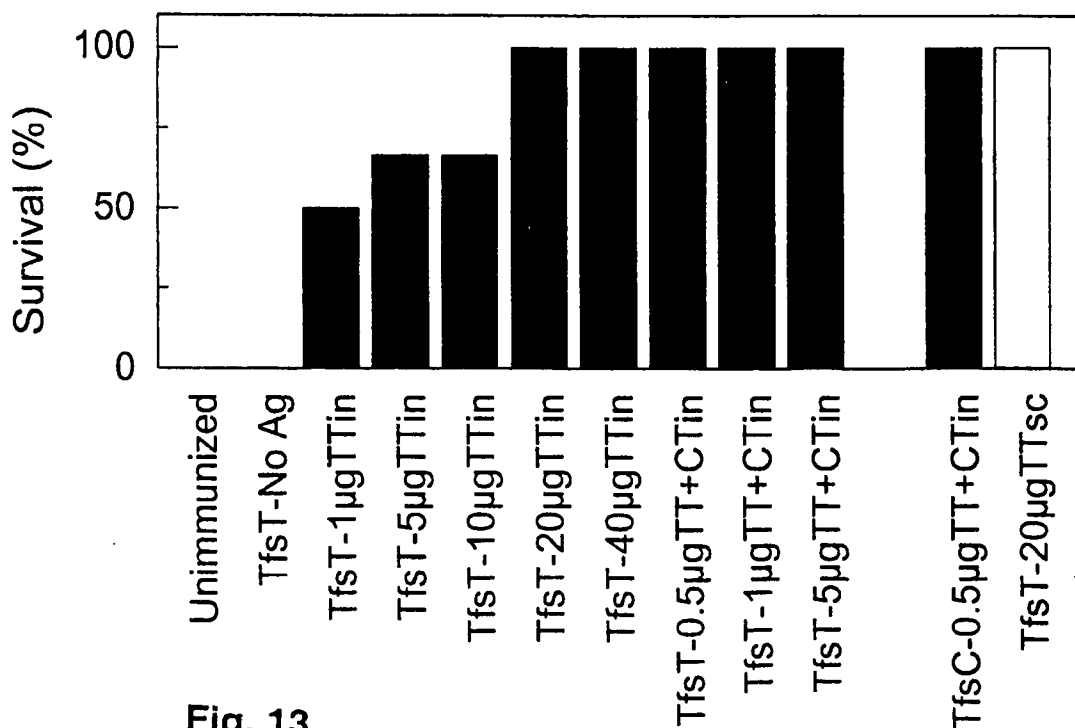
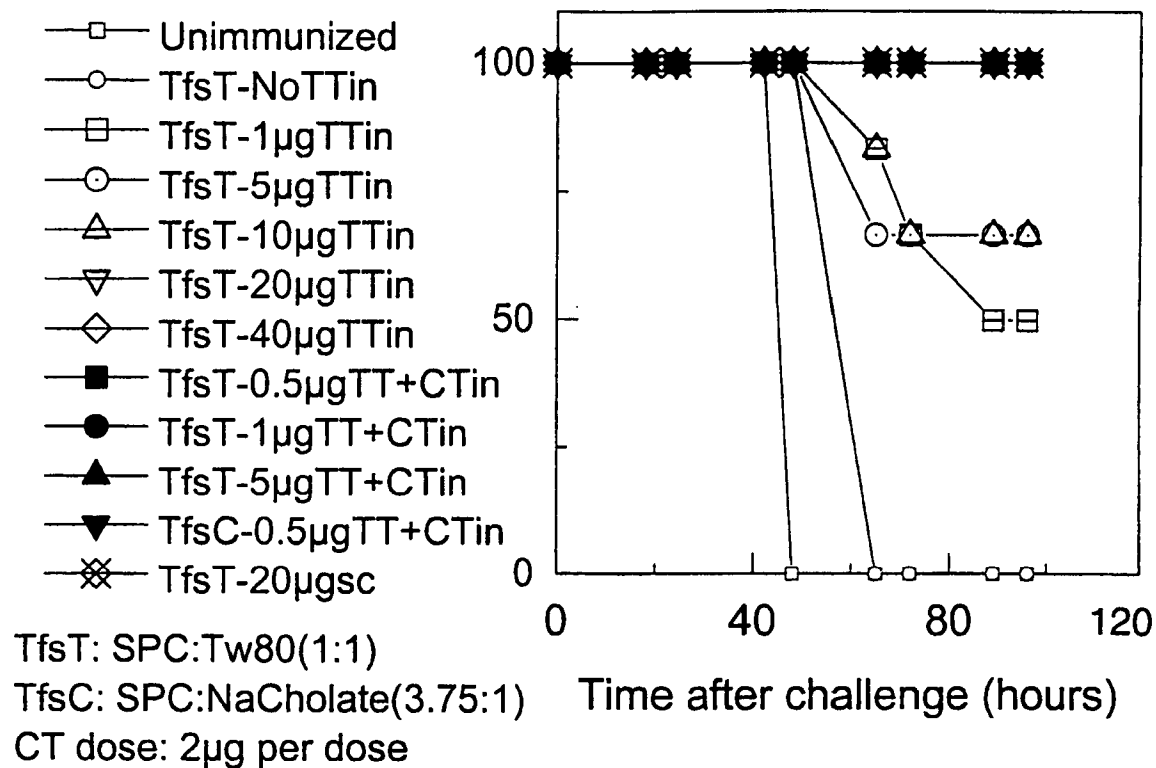


Fig. 13

Adjuvant effect: of Heat Labile Toxin (LT) from E.coli

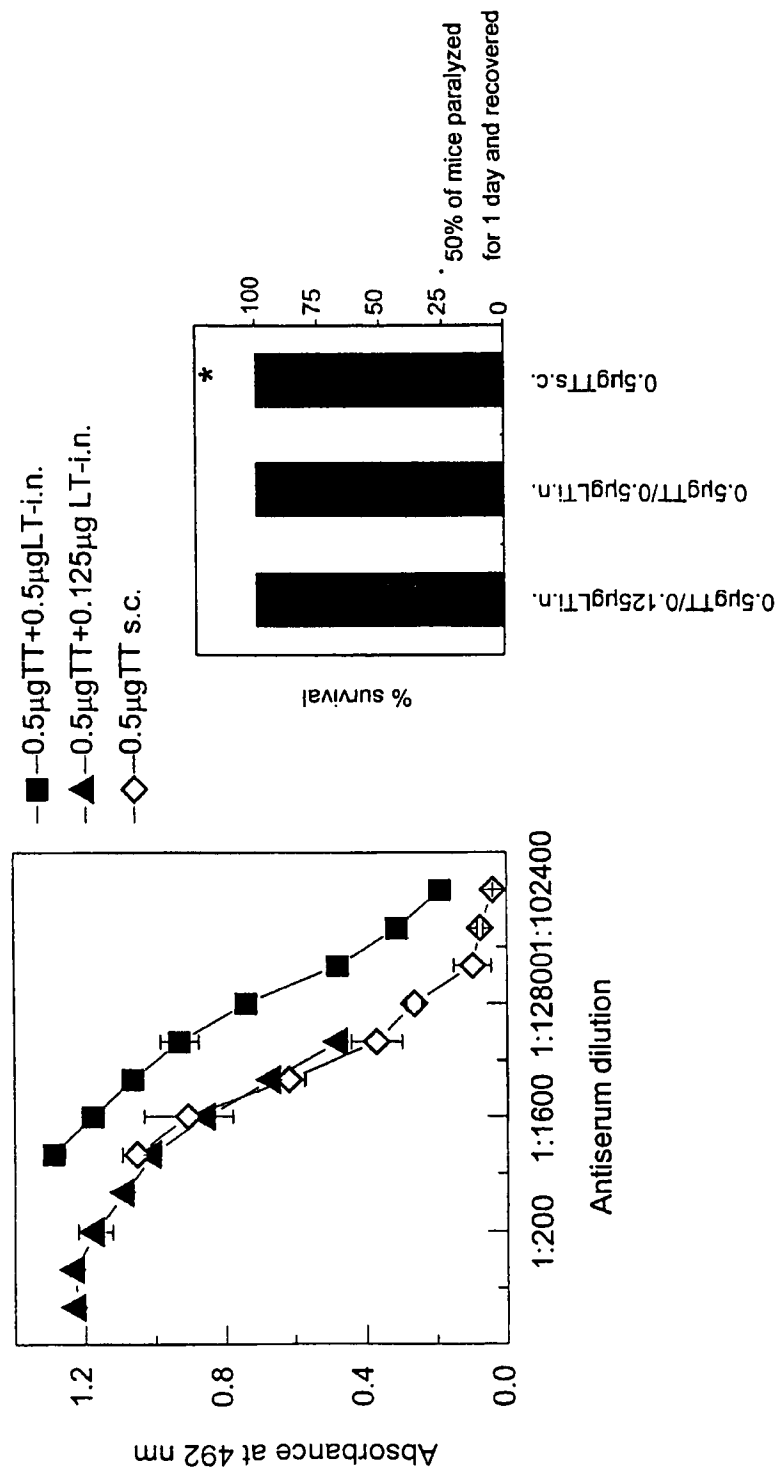


Fig. 14

Bi-Valent Vaccines: Anti-Tetanus and -Cholera response to the antigens administration in Transfersomes in the nose

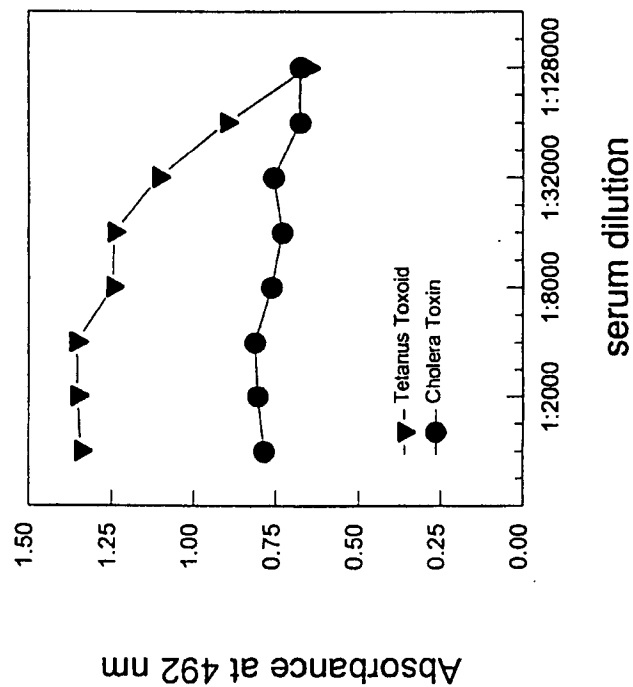


Fig. 15

INTERNATIONAL SEARCH REPORT

Internat Application No

PCT/EP 00/00598

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K9/127 A61K38/19 A61K39/39 A61K38/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 475 160 A (CEVC GREGOR) 18 March 1992 (1992-03-18) cited in the application page 1, line 1-6 examples claims — -/--	4,5

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	<p>WO 92 04009 A (UNIV LONDON PHARMACY) 19 March 1992 (1992-03-19) page 1, line 3-7 page 3, line 21-32 page 6-7; example 1 page 14; example 2 claims</p>	1-53
A	<p>CEVC G: "Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery" CRITICAL REVIEWS IN THERAPEUTIC DRUG CARRIER SYSTEMS, vol. 13, no. 3-4, 1996, pages 257-388, XP002107366 page 316 -page 321</p>	1

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information on patent family members

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(54) Title: COMPOSITIONS AND METHODS FOR IMPROVED DELIVERY OF HYDROPHOBIC THERAPEUTIC AGENTS		
(57) Abstract <p>The present invention relates to triglyceride-free pharmaceutical compositions for delivery of hydrophobic therapeutic agents. Compositions of the present invention include a hydrophobic therapeutic agent and a carrier, where the carrier is formed from a combination of a hydrophilic surfactant and a hydrophobic surfactant. Upon dilution with an aqueous solvent, the composition forms a clear, aqueous dispersion of the surfactants containing the therapeutic agent. The invention also provides methods of treatment with hydrophobic therapeutic agents using these compositions.</p>		

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1 **COMPOSITIONS AND METHODS FOR IMPROVED DELIVERY
OF HYDROPHOBIC THERAPEUTIC AGENTS**

FIELD OF THE INVENTION

5 The present invention relates to drug delivery systems, and in particular to pharmaceutical compositions for the improved delivery of hydrophobic compounds.

BACKGROUND

 Hydrophobic therapeutic agents, *i.e.*, therapeutic compounds having poor solubility in aqueous solution, present difficult problems in formulating such compounds for effective
10 administration to patients. A well-designed formulation must, at a minimum, be capable of presenting a therapeutically effective amount of the hydrophobic compound to the desired absorption site, in an absorbable form. Even this minimal functionality is difficult to achieve when delivery of the hydrophobic therapeutic agent requires interaction with aqueous physiological environments, such as gastric fluids and intestinal fluids. Pharmaceutical
15 compositions for delivery of such hydrophobic therapeutic agents must carry the hydrophobic compound through the aqueous environment, while maintaining the hydrophobic compound in an absorbable form, and avoiding the use of physiologically harmful solvents or excipients.

 A number of approaches to formulating hydrophobic therapeutic agents for oral or parenteral delivery are known. One well-known approach uses surfactant micelles to
20 solubilize and transport the therapeutic agent. Micelles are agglomerates of colloidal dimensions formed by amphiphilic compounds under certain conditions. Micelles, and pharmaceutical compositions containing micelles, have been extensively studied and are described in detail in the literature; *see, e.g.*, Remington's Pharmaceutical Sciences, 17th ed. (1985), the disclosure of which is incorporated herein in its entirety. In aqueous solution,
25 micelles can incorporate hydrophobic therapeutic agents in the hydrocarbon core of the micelle, or entangled at various positions within the micelle walls. Although micellar formulations can solubilize a variety of hydrophobic therapeutic agents, the loading capacity of conventional micelle formulations is limited by the solubility of the therapeutic agent in the micelle surfactant. For many hydrophobic therapeutic agents, such solubility is too low to
30 offer formulations that can deliver therapeutically effective doses.

 Another conventional approach takes advantage of the increased solubility of hydrophobic therapeutic agents in oils (triglycerides). Hydrophobic therapeutic agents, while poorly soluble in aqueous solution, could be sufficiently lipophilic that therapeutically

1 effective concentrations of the therapeutic agents can be prepared in triglyceride-based
solvents. Thus, one conventional approach is to solubilize a hydrophobic therapeutic agent in
a bioacceptable triglyceride solvent, such as a digestible vegetable oil, and disperse this oil
phase in an aqueous solution. The dispersion may be stabilized by emulsifying agents and
5 provided in emulsion form. Alternatively, the therapeutic agent can be provided in a water-
free formulation, with an aqueous dispersion being formed in the in vivo gastrointestinal
environment. The properties of these oil-based formulations are determined by such factors
as the size of the triglyceride/therapeutic agent colloidal particles and the presence or absence
of surfactant additives.

10 In simplest form, a triglyceride-containing formulation suitable for delivering
hydrophobic therapeutic agents through an aqueous environment is an oil-in-water emulsion.
Such emulsions contain the hydrophobic therapeutic agent solubilized in an oil phase which
is dispersed in an aqueous environment with the aid of a surfactant. The surfactant may be
present in the oil-based formulation itself, or may be a compound provided in the
15 gastrointestinal system, such as bile salts, which are known to be in vivo emulsifying agents.
The colloidal oil particles sizes are relatively large, ranging from several hundred nanometers
to several microns in diameter, in a broad particle size distribution. Since the particle sizes
are on the order of or greater than the wavelength range of visible light, such emulsions,
when prepared in an emulsion dosage form, are visibly "cloudy" or "milky" to the naked eye.

20 Although triglyceride-based pharmaceutical compositions are useful in solubilizing
and delivering some hydrophobic therapeutic agents, such compositions are subject to a
number of significant limitations and disadvantages. Emulsions are thermodynamically
unstable, and colloidal emulsion particles will spontaneously agglomerate, eventually leading
to complete phase separation. The tendency to agglomerate and phase separate presents
25 problems of storage and handling, and increases the likelihood that pharmaceutical emulsions
initially properly prepared will be in a less optimal, less effective, and poorly-characterized
state upon ultimate administration to a patient. Uncharacterized degradation is particularly
disadvantageous, since increased particle size slows the rate of transport of the colloidal
particle and digestion of the oil component, and hence the rate and extent of absorption of the
30 therapeutic agent. These problems lead to poorly-characterized and potentially harmful
changes in the effective dosage received by the patient. Moreover, changes in colloidal
emulsion particle size are also believed to render absorption more sensitive to and dependent
upon conditions in the gastrointestinal tract, such as pH, enzyme activity, bile components,

1 and stomach contents. Such uncertainty in the rate and extent of ultimate absorption of the therapeutic agent severely compromises the medical professional's ability to safely administer therapeutically effective dosages.

5 A further disadvantage of triglyceride-containing compositions is the dependence of therapeutic agent absorption on the rate and extent of lipolysis. Although colloidal emulsion particles can transport hydrophobic therapeutic agents through the aqueous environment of the gastrointestinal tract, ultimately the triglyceride must be digested and the therapeutic agent must be released in order to be absorbed through the intestinal mucosa. The triglyceride carrier is emulsified by bile salts and hydrolyzed, primarily by pancreatic lipase. 10 The rate and extent of lipolysis, however, are dependent upon several factors that are difficult to adequately control. For example, the amount and rate of bile salt secretion affect the lipolysis of the triglycerides, and the bile salt secretion can vary with stomach contents, with metabolic abnormalities, and with functional changes of the liver, bile ducts, gall bladder and intestine. Lipase availability in patients with decreased pancreatic secretory function, such as 15 cystic fibrosis or chronic pancreatitis, may be undesirably low, resulting in a slow and incomplete triglyceride lipolysis. The activity of lipase is pH dependent, with deactivation occurring at about pH 3, so that the lipolysis rate will vary with stomach contents, and may be insufficient in patients with gastric acid hyper-secretion. Moreover, certain surfactants commonly used in the preparation of pharmaceutical emulsions, such as polyethoxylated 20 castor oils, may themselves act as inhibitors of lipolysis. Although recent work suggests that certain surfactant combinations, when used in combination with digestible oils in emulsion preparations, can substantially decrease the lipolysis-inhibiting effect of some common pharmaceutical surfactants (*see*, U.S. Patent No. 5,645,856), such formulations are still subject to the other disadvantages of pharmaceutical emulsions and triglyceride-based 25 formulations.

Yet another approach is based on formation of "microemulsions." Like an emulsion, a microemulsion is a liquid dispersion of oil in water, stabilized by surfactants. The microemulsion particles are smaller than those of an emulsion, rendering the microemulsion essentially optically clear. Microemulsions, however, are thermodynamically stable, and are 30 not subject to the particle agglomeration problems of conventional emulsions. It is generally believed that microemulsions are micelle-like particles, having an essentially micellar structure but containing a distinct oil phase in the micelle "core". These micelle-like particles are often referred to as "swollen micelles", a term which emphasizes their close relationship

1 to true micellar particles. Despite their close relationship to micelles, microemulsions
function quite differently in drug delivery systems. The majority of hydrophobic therapeutic
agents are lipophilic, and have greater solubility in triglycerides than in surfactants. As a
5 result, the hydrophobic therapeutic agent in a microemulsion-based delivery system is
preferentially solvated in the triglyceride phase, which is in turn encapsulated in the swollen
micelle. The preferential partitioning in the triglyceride phase results in higher loading
capacities than in comparable micelle-based systems, but at the cost of introducing into the
delivery system the lipolysis-dependence and other disadvantages associated with the
10 presence of triglycerides. In addition, the larger size of microemulsion particles, relative to
true micelles, results in a slower rate of particle diffusion, and thus a slower rate of
therapeutic agent absorption.

Thus, there is a need for pharmaceutical compositions that overcome the limitations
of conventional micelle formulations, but without suffering from the disadvantages of
triglyceride-containing formulations.

15 SUMMARY OF THE INVENTION

The present invention provides pharmaceutical compositions for improved delivery of
hydrophobic therapeutic agents. In one embodiment, the present invention provides a
triglyceride-free pharmaceutical composition including a hydrophobic therapeutic agent and a
carrier. The carrier includes a hydrophilic surfactant and a hydrophobic surfactant in
20 amounts such that upon dilution with an aqueous solution such as simulated gastrointestinal
fluids the carrier forms a clear aqueous dispersion of the hydrophilic and hydrophobic
surfactants containing the hydrophobic therapeutic agent.

In another embodiment, the present invention provides a clear aqueous dispersion
containing a hydrophilic surfactant, a hydrophobic surfactant and a hydrophobic therapeutic
25 agent. The dispersion is substantially free of triglycerides.

In another embodiment, the present invention relates to a triglyceride-free
pharmaceutical composition which includes a hydrophilic surfactant and a hydrophobic
surfactant in amounts such that upon dilution with an aqueous solution a clear aqueous
dispersion is formed, a first amount of a hydrophobic therapeutic agent solubilized in the
30 clear aqueous dispersion, and a second amount of the hydrophobic therapeutic agent that
remains non-solubilized but dispersed.

1 In another embodiment, the present invention relates to methods of increasing the rate and/or extent of absorption of hydrophobic therapeutic agents by administering to a patient a pharmaceutical composition of the present invention.

5 These features of the present invention will become more fully apparent from the following description and appended claims, or may be learned by the practice of the invention as set forth hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

10 In order to illustrate the manner in which the above-recited and other advantages and objects of the invention are obtained, a more particular description of the invention briefly described above will be rendered by reference to the specific embodiments shown in the appended drawings. Understanding that these drawings depict only typical embodiments of the invention and are not therefore limiting of its scope, the invention will be described and explained with additional specificity and detail through the use of the accompanying drawing, in which:

15 Figure 1 shows the enhanced bioabsorption of a hydrophobic therapeutic agent in the compositions of the present invention, relative to a commercial formulation.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

20 The present invention overcomes the problems described above characteristic of conventional formulations such as micelle formulations, emulsions, and microemulsions, by providing unique triglyceride-free pharmaceutical compositions. Surprisingly, the present inventors have found that compositions including a combination of a hydrophilic surfactant and a hydrophobic surfactant can solubilize therapeutically effective amounts of hydrophobic therapeutic agents without recourse to the use of triglycerides, thereby avoiding the lipolysis dependence and other disadvantages of conventional formulations. Use of these formulations results in an enhanced rate and/or extent of absorption of the hydrophobic therapeutic agent.

A. Pharmaceutical Compositions

30 In one embodiment, the present invention provides a pharmaceutical composition including a carrier and a hydrophobic therapeutic agent. The carrier includes a hydrophilic surfactant and a hydrophobic surfactant in amounts such that upon dilution with an aqueous solution the carrier forms a clear aqueous dispersion of the hydrophilic and hydrophobic surfactants containing the hydrophobic therapeutic agent. It is a particular feature of the present invention that the carrier is substantially free of triglycerides, thereby providing surprising and important advantages over conventional, triglyceride-containing formulations.

1. Surfactants

The carrier includes at least one hydrophilic surfactant and at least one hydrophobic surfactant. As is well known in the art, the terms "hydrophilic" and "hydrophobic" are relative terms. To function as a surfactant, a compound must necessarily include polar or charged hydrophilic moieties as well as non-polar hydrophobic (lipophilic) moieties; *i.e.*, a surfactant compound must be amphiphilic. An empirical parameter commonly used to characterize the relative hydrophilicity and hydrophobicity of non-ionic amphiphilic compounds is the hydrophilic-lipophilic balance ("HLB" value). Surfactants with lower HLB values are more hydrophobic, and have greater solubility in oils, while surfactants with higher HLB values are more hydrophilic, and have greater solubility in aqueous solutions.

Using HLB values as a rough guide, hydrophilic surfactants are generally considered to be those compounds having an HLB value greater than about 10, as well as anionic, cationic, or zwitterionic compounds for which the HLB scale is not generally applicable. Similarly, hydrophobic surfactants are compounds having an HLB value less than about 10.

It should be appreciated that the HLB value of a surfactant is merely a rough guide generally used to enable formulation of industrial, pharmaceutical and cosmetic emulsions. For many important surfactants, including several polyethoxylated surfactants, it has been reported that HLB values can differ by as much as about 8 HLB units, depending upon the empirical method chosen to determine the HLB value (Schott, *J. Pharm. Sciences*, 79(1), 87-88 (1990)). Likewise, for certain polypropylene oxide containing block copolymers (PLURONIC® surfactants, BASF Corp.), the HLB values may not accurately reflect the true physical chemical nature of the compounds. Finally, commercial surfactant products are generally not pure compounds, but are complex mixtures of compounds, and the HLB value reported for a particular compound may more accurately be characteristic of the commercial product of which the compound is a major component. Different commercial products having the same primary surfactant component can, and typically do, have different HLB values. In addition, a certain amount of lot-to-lot variability is expected even for a single commercial surfactant product. Keeping these inherent difficulties in mind, and using HLB values as a guide, one skilled in the art can readily identify surfactants having suitable hydrophilicity or hydrophobicity for use in the present invention, as described herein.

The hydrophilic surfactant can be any hydrophilic surfactant suitable for use in pharmaceutical compositions. Such surfactants can be anionic, cationic, zwitterionic or non-ionic, although non-ionic hydrophilic surfactants are presently preferred. As discussed

1 above, these non-ionic hydrophilic surfactants will generally have HLB values greater than about 10. Mixtures of hydrophilic surfactants are also within the scope of the invention.

Similarly, the hydrophobic surfactant can be any hydrophobic surfactant suitable for use in pharmaceutical compositions. In general, suitable hydrophobic surfactants will have
5 an HLB value less than about 10. Mixtures of hydrophobic surfactants are also within the scope of the invention.

The choice of specific hydrophobic and hydrophilic surfactants should be made keeping in mind the particular hydrophobic therapeutic agent to be used in the composition, and the range of polarity appropriate for the chosen therapeutic agent, as discussed in more
10 detail below. With these general principles in mind, a very broad range of surfactants is suitable for use in the present invention. Such surfactants can be grouped into the following general chemical classes detailed in the Tables below. The HLB values given in the Tables below generally represent the HLB value as reported by the manufacturer of the
15 corresponding commercial product. In cases where more than one commercial product is listed, the HLB value in the Tables is the value as reported for one of the commercial products, a rough average of the reported values, or a value that, in the judgment of the present inventors, is more reliable. It should be emphasized that the invention is not limited to the surfactants in the following Tables, which show representative, but not exclusive, lists of available surfactants.

20 1.1. Polyethoxylated Fatty Acids

Although polyethylene glycol (PEG) itself does not function as a surfactant, a variety of PEG-fatty acid esters have useful surfactant properties. Among the PEG-fatty acid monoesters, esters of lauric acid, oleic acid, and stearic acid are most useful. Among the
25 surfactants of Table 1, preferred hydrophilic surfactants include PEG-8 laurate, PEG-8 oleate, PEG-8 stearate, PEG-9 oleate, PEG-10 laurate, PEG-10 oleate, PEG-12 laurate, PEG-12 oleate, PEG-15 oleate, PEG-20 laurate and PEG-20 oleate. Examples of polyethoxylated fatty acid monoester surfactants commercially available are shown in Table 1.

Table 1: PEG-Fatty Acid Monoester Surfactants

COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
30 PEG 4-100 monolaurate	Crodet L series (Croda)	>9
PEG 4-100 monooleate	Crodet O series (Croda)	>8
PEG 4-100 monostearate	Crodet S series (Croda), Myrj Series (Atlas/ICI)	>6

1	PEG 400 distearate	Cithrol 4DS series (Croda)	>10
	PEG 100,200,300 monolaurate	Cithrol ML series (Croda)	>10
5	PEG 100,200,300 monooleate	Cithrol MO series (Croda)	>10
	PEG 400 dioleate	Cithrol 4DO series (Croda)	>10
	PEG 400-1000 monostearate	Cithrol MS series (Croda)	>10
10	PEG-1 stearate	Nikkol MYS-1EX (Nikko), Coster K1 (Condea)	2
	PEG-2 stearate	Nikkol MYS-2 (Nikko)	4
	PEG-2 oleate	Nikkol MYO-2 (Nikko)	4.5
	PEG-4 laurate	Mapeg® 200 ML (PPG), Kessco® PEG 200ML (Stepan), LIPOPEG 2L (LIPO Chem.)	9.3
15	PEG-4 oleate	Mapeg® 200 MO (PPG), Kessco® PEG200 MO (Stepan),	8.3
	PEG-4 stearate	Kessco® PEG 200 MS (Stepan), Hodag 20 S (Calgene), Nikkol MYS-4 (Nikko)	6.5
	PEG-5 stearate	Nikkol TMGS-5 (Nikko)	9.5
	PEG-5 oleate	Nikkol TMGO-5 (Nikko)	9.5
20	PEG-6 oleate	Algon OL 60 (Auschem SpA), Kessco® PEG 300 MO (Stepan), Nikkol MYO-6 (Nikko), Emulgante A6 (Condea)	8.5
	PEG-7 oleate	Algon OL 70 (Auschem SpA)	10.4
	PEG-6 laurate	Kessco® PEG300 ML (Stepan)	11.4
25	PEG-7 laurate	Lauridac 7 (Condea)	13
	PEG-6 stearate	Kessco® PEG300 MS (Stepan)	9.7
	PEG-8 laurate	Mapeg® 400 ML (PPG), LIPOPEG 4DL(Lipo Chem.)	13
	PEG-8 oleate	Mapeg® 400 MO (PPG), Emulgante A8 (Condea)	12
30	PEG-8 stearate	Mapeg® 400 MS (PPG), Myrj 45	12
	PEG-9 oleate	Emulgante A9 (Condea)	>10
	PEG-9 stearate	Cremophor S9 (BASF)	>10

1	PEG-10 laurate	Nikkol MYL-10 (Nikko), Lauridac 10 (Croda)	13
	PEG-10 oleate	Nikkol MYO-10 (Nikko)	11
	PEG-10 stearate	Nikkol MYS-10 (Nikko), Coster K100 (Condea)	11
5	PEG-12 laurate	Kessco® PEG 600ML (Stepan)	15
	PEG-12 oleate	Kessco® PEG 600MO (Stepan)	14
	PEG-12 ricinoleate	(CAS # 9004-97-1)	>10
	PEG-12 stearate	Mapeg® 600 MS (PPG), Kessco® PEG 600MS (Stepan)	14
10	PEG-15 stearate	Nikkol TMGS-15 (Nikko), Koster K15 (Condea)	14
	PEG-15 oleate	Nikkol TMGO-15 (Nikko)	15
	PEG-20 laurate	Kessco® PEG 1000 ML (Stepan)	17
	PEG-20 oleate	Kessco® PEG 1000 MO (Stepan)	15
	PEG-20 stearate	Mapeg® 1000 MS (PPG), Kessco® PEG 1000 MS (Stepan), Myrj 49	16
15	PEG-25 stearate	Nikkol MYS-25 (Nikko)	15
	PEG-32 laurate	Kessco® PEG 1540 ML (Stepan)	16
	PEG-32 oleate	Kessco® PEG 1540 MO (Stepan)	17
	PEG-32 stearate	Kessco® PEG 1540 MS (Stepan)	17
20	PEG-30 stearate	Myrj 51	>10
	PEG-40 laurate	Crodet L40 (Croda)	17.9
	PEG-40 oleate	Crodet O40 (Croda)	17.4
	PEG-40 stearate	Myrj 52, Emerest® 2715 (Henkel), Nikkol MYS-40 (Nikko)	>10
25	PEG-45 stearate	Nikkol MYS-45 (Nikko)	18
	PEG-50 stearate	Myrj 53	>10
	PEG-55 stearate	Nikkol MYS-55 (Nikko)	18
	PEG-100 oleate	Crodet O-100 (Croda)	18.8
30	PEG-100 stearate	Myrj 59, Arlacel 165 (ICI)	19
	PEG-200 oleate	Albunol 200 MO (Taiwan Surf.)	>10
	PEG-400 oleate	LACTOMUL (Henkel), Albunol 400 MO (Taiwan Surf.)	>10

1	PEG-600 oleate	Albunol 600 MO (Taiwan Surf.)	>10
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1.2 PEG-Fatty Acid Diesters

5 Polyethylene glycol fatty acid diesters are also suitable for use as surfactants in the compositions of the present invention. Among the surfactants in Table 2, preferred hydrophilic surfactants include PEG-20 dilaurate, PEG-20 dioleate, PEG-20 distearate, PEG-32 dilaurate and PEG-32 dioleate. Representative PEG-fatty acid diesters are shown in Table 2.

10	Table 2: PEG-Fatty Acid Diester Surfactants		
	COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
	PEG-4 dilaurate	Mapeg® 200 DL (PPG), Kessco® PEG 200 DL (Stepan), LIPOPEG 2-DL (Lipo Chem.)	7
	PEG-4 dioleate	Mapeg® 200 DO (PPG),	6
15	PEG-4 distearate	Kessco® 200 DS (Stepan_	5
	PEG-6 dilaurate	Kessco® PEG 300 DL (Stepan)	9.8
	PEG-6 dioleate	Kessco® PEG 300 DO (Stepan)	7.2
	PEG-6 distearate	Kessco® PEG 300 DS (Stepan)	6.5
20	PEG-8 dilaurate	Mapeg® 400 DL (PPG), Kessco® PEG 400 DL (Stepan), LIPOPEG 4 DL (Lipo Chem.)	11
	PEG-8 dioleate	Mapeg® 400 DO (PPG), Kessco® PEG 400 DO (Stepan), LIPOPEG 4 DO(Lipo Chem.)	8.8
	PEG-8 distearate	Mapeg® 400 DS (PPG), CDS 400 (Nikkol)	11
	PEG-10 dipalmitate	Polyaldo 2PKFG	>10
25	PEG-12 dilaurate	Kessco® PEG 600 DL (Stepan)	11.7
	PEG-12 distearate	Kessco® PEG 600 DS (Stepan)	10.7
	PEG-12 dioleate	Mapeg® 600 DO (PPG), Kessco® 600 DO(Stepan)	10
	PEG-20 dilaurate	Kessco® PEG 1000 DL (Stepan)	15
30	PEG-20 dioleate	Kessco® PEG 1000 DO (Stepan)	13
	PEG-20 distearate	Kessco® PEG 1000 DS (Stepan)	12
	PEG-32 dilaurate	Kessco® PEG 1540 DL (Stepan)	16

1	PEG-32 dioleate	Kessco® PEG 1540 DO (Stepan)	15
	PEG-32 distearate	Kessco® PEG 1540 DS (Stepan)	15
	PEG-400 dioleate	Cithrol 4DO series (Croda)	>10
5	PEG-400 distearate	Cithrol 4DS series (Croda)	>10

1.3 PEG-Fatty Acid Mono- and Di-ester Mixtures

In general, mixtures of surfactants are also useful in the present invention, including mixtures of two or more commercial surfactant products. Several PEG-fatty acid esters are marketed commercially as mixtures or mono- and diesters. Representative surfactant mixtures are shown in Table 3.

Table 3: PEG-Fatty Acid Mono- and Diester Mixtures

COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
15 PEG 4-150 mono, dilaurate	Kessco® PEG 200-6000 mono, dilaurate (Stepan)	
PEG 4-150 mono, dioleate	Kessco® PEG 200-6000 mono, dioleate (Stepan)	
PEG 4-150 mono, distearate	Kessco® 200-6000 mono, distearate (Stepan)	

20 1.4 Polyethylene Glycol Glycerol Fatty Acid Esters

Suitable PEG glycerol fatty acid esters are shown in Table 4. Among the surfactants in the Table, preferred hydrophilic surfactants are PEG-20 glyceryl laurate, PEG-30 glyceryl laurate, PEG-40 glyceryl laurate, PEG-20 glyceryl oleate, and PEG-30 glyceryl oleate.

Table 4: PEG Glycerol Fatty Acid Esters

COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
25 PEG-20 glyceryl laurate	Tagat® L (Goldschmidt)	16
PEG-30 glyceryl laurate	Tagat® L2 (Goldschmidt)	16
PEG-15 glyceryl laurate	Glycerox L series (Croda)	15
30 PEG-40 glyceryl laurate	Glycerox L series (Croda)	15
PEG-20 glyceryl stearate	Capmul® EMG (ABITEC), Aldo® MS-20 KFG (Lonza)	13
PEG-20 glyceryl oleate	Tagat® O (Goldschmidt)	>10
PEG-30 glyceryl oleate	Tagat® O2 (Goldschmidt)	>10

1.5. Alcohol - Oil Transesterification Products

A large number of surfactants of different degrees of hydrophobicity or hydrophilicity can be prepared by reaction of alcohols or polyalcohols with a variety of natural and/or hydrogenated oils. Most commonly, the oils used are castor oil or hydrogenated castor oil, or an edible vegetable oil such as corn oil, olive oil, peanut oil, palm kernel oil, apricot kernel oil, or almond oil. Preferred alcohols include glycerol, propylene glycol, ethylene glycol, polyethylene glycol, sorbitol, and pentaerythritol. Among these alcohol-oil transesterified surfactants, preferred hydrophilic surfactants are PEG-35 castor oil (Incrocas-35), PEG-40 hydrogenated castor oil (Cremophor RH 40), PEG-25 trioleate (TAGAT® TO), PEG-60 corn glycerides (Crovol M70), PEG-60 almond oil (Crovol A70), PEG-40 palm kernel oil (Crovol PK70), PEG-50 castor oil (Emalex C-50), PEG-50 hydrogenated castor oil (Emalex HC-50), PEG-8 caprylic/capric glycerides (Labrasol), and PEG-6 caprylic/capric glycerides (Softigen 767). Preferred hydrophobic surfactants in this class include PEG-5 hydrogenated castor oil, PEG-7 hydrogenated castor oil, PEG-9 hydrogenated castor oil, PEG-6 corn oil (Labrafil® M 2125 CS), PEG-6 almond oil (Labrafil® M 1966 CS), PEG-6 apricot kernel oil (Labrafil® M 1944 CS), PEG-6 olive oil (Labrafil® M 1980 CS), PEG-6 peanut oil (Labrafil® M 1969 CS), PEG-6 hydrogenated palm kernel oil (Labrafil® M 2130 BS), PEG-6 palm kernel oil (Labrafil® M 2130 CS), PEG-6 triolein (Labrafil® M 2735 CS), PEG-8 corn oil (Labrafil® WL 2609 BS), PEG-20 corn glycerides (Crovol M40), and PEG-20 almond glycerides (Crovol A40). The latter two surfactants are reported to have HLB values of 10, which is generally considered to be the approximate border line between hydrophilic and hydrophobic surfactants. For purposes of the present invention, these two surfactants are considered to be hydrophobic. Representative surfactants of this class suitable for use in the present invention are shown in Table 5.

Table 5: Transesterification Products of Oils and Alcohols

COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
PEG-3 castor oil	Nikkol CO-3 (Nikko)	3
PEG-5, 9, and 16 castor oil	ACCONON CA series (ABITEC)	6-7
PEG-20 castor oil	Emalex C-20 (Nihon Emulsion), Nikkol CO-20 TX (Nikko)	11
PEG-23 castor oil	Emulgante EL23	>10

1	PEG-30 castor oil	Emalex C-30 (Nihon Emulsion), Alkamuls® EL 620 (Rhone-Poulenc), Incrocas 30 (Croda)	11
	PEG-35 castor oil	Cremophor EL and EL-P (BASF), Emulphor EL, Incrocas-35 (Croda), Emulgin RO 35 (Henkel)	
5	PEG-38 castor oil	Emulgante EL 65 (Condea)	
	PEG-40 castor oil	Emalex C-40 (Nihon Emulsion), Alkamuls® EL 719 (Rhone-Poulenc)	13
	PEG-50 castor oil	Emalex C-50 (Nihon Emulsion)	14
	PEG-56 castor oil	Eumulgin® PRT 56 (Pulcra SA)	>10
10	PEG-60 castor oil	Nikkol CO-60TX (Nikko)	14
	PEG-100 castor oil	Thornley	>10
	PEG-200 castor oil	Eumulgin® PRT 200 (Pulcra SA)	>10
	PEG-5 hydrogenated castor oil	Nikkol HCO-5 (Nikko)	6
15	PEG-7 hydrogenated castor oil	Simusol® 989 (Seppic), Cremophor WO7 (BASF)	6
	PEG-10 hydrogenated castor oil	Nikkol HCO-10 (Nikko)	6.5
20	PEG-20 hydrogenated castor oil	Nikkol HCO-20 (Nikko)	11
	PEG-25 hydrogenated castor oil	Simulsol® 1292 (Seppic), Cerex ELS 250 (Auschem SpA)	11
	PEG-30 hydrogenated castor oil	Nikkol HCO-30 (Nikko)	11
25	PEG-40 hydrogenated castor oil	Cremophor RH 40 (BASF), Croduret (Croda), Emulgin HRE 40 (Henkel)	13
	PEG-45 hydrogenated castor oil	Cerex ELS 450 (Auschem Spa)	14
	PEG-50 hydrogenated castor oil	Emalex HC-50 (Nihon Emulsion)	14
30	PEG-60 hydrogenated castor oil	Nikkol HCO-60 (Nikko); Cremophor RH 60 (BASF)	15
	PEG-80 hydrogenated castor oil	Nikkol HCO-80 (Nikko)	15

1	PEG-100 hydrogenated castor oil	Nikkol HCO -100 (Nikko)	17
	PEG-6 corn oil	Labrafil® M 2125 CS (Gattefosse)	4
	PEG-6 almond oil	Labrafil® M 1966 CS (Gattefosse)	4
5	PEG-6 apricot kernel oil	Labrafil® M 1944 CS (Gattefosse)	4
	PEG-6 olive oil	Labrafil® M 1980 CS (Gattefosse)	4
	PEG-6 peanut oil	Labrafil® M 1969 CS (Gattefosse)	4
	PEG-6 hydrogenated palm kernel oil	Labrafil® M 2130 BS (Gattefosse)	4
10	PEG-6 palm kernel oil	Labrafil® M 2130 CS (Gattefosse)	4
	PEG-6 triolein	Labrafil® M 2735 CS (Gattefosse)	4
	PEG-8 corn oil	Labrafil® WL 2609 BS (Gattefosse)	6-7
	PEG-20 corn glycerides	Crovol M40 (Croda)	10
15	PEG-20 almond glycerides	Crovol A40 (Croda)	10
	PEG-25 trioleate	TAGAT® TO (Goldschmidt)	11
	PEG-40 palm kernel oil	Crovol PK-70	>10
	PEG-60 corn glycerides	Crovol M70 (Croda)	15
20	PEG-60 almond glycerides	Crovol A70 (Croda)	15
	PEG-4 caprylic/capric triglyceride	Labrafac® Hydro (Gattefosse),	4-5
	PEG-8 caprylic/capric glycerides	Labrasol (Gattefosse), Labrafac CM 10 (Gattefosse)	>10
25	PEG-6 caprylic/capric glycerides	SOFTIGEN® 767 (Hüls), Glycerox 767 (Croda)	19
	Lauroyl macrogol-32 glyceride	GELUCIRE 44/14 (Gattefosse)	14
	Stearoyl macrogol glyceride	GELUCIRE 50/13 (Gattefosse)	13
30	Mono, di, tri, tetra esters of Sorbitol vegetable oils and sorbitol	Glyceride (Gattefosse)	<10

1	Pentaerythrityl tetraisostearate	Crodamol PTIS (Croda)	<10
	Pentaerythrityl distearate	Albunol DS (Taiwan Surf.)	<10
5	Pentaerythrityl tetraoleate	Liponate PO-4 (Lipo Chem.)	<10
	Pentaerythrityl tetrastearate	Liponate PS-4 (Lipo Chem.)	<10
	Pentaerythrityl tetracaprylate/tetracaprat e	Liponate PE-810 (Lipo Chem.), Crodamol PTC (Croda)	<10
10	Pentaerythrityl tetraoctanoate	Nikkol Pentarate 408 (Nikko)	

Also included as oils in this category of surfactants are oil-soluble vitamins, such as vitamins A, D, E, K, etc. Thus, derivatives of these vitamins, such as tocopheryl PEG-1000 succinate (TPGS, available from Eastman), are also suitable surfactants.

15 1.6. Polyglycerized Fatty Acids

Polyglycerol esters of fatty acids are also suitable surfactants for the present invention. Among the polyglyceryl fatty acid esters, preferred hydrophobic surfactants include polyglyceryl oleate (Plurol Oleique), polyglyceryl-2 dioleate (Nikkol DGDO), and polyglyceryl-10 trioleate. Preferred hydrophilic surfactants include polyglyceryl-10 laurate (Nikkol Decaglyn 1-L), polyglyceryl-10 oleate (Nikkol Decaglyn 1-O), and polyglyceryl-10
20 mono, dioleate (Caprol® PEG 860). Polyglyceryl polyricinoleates (Polymuls) are also preferred hydrophilic and hydrophobic surfactants. Examples of suitable polyglyceryl esters are shown in Table 6.

Table 6: Polyglycerized Fatty Acids

25	COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
	Polyglyceryl-2 stearate	Nikkol DGMS (Nikko)	5-7
	Polyglyceryl-2 oleate	Nikkol DGMO (Nikko)	5-7
	Polyglyceryl-2 isostearate	Nikkol DGMIS (Nikko)	5-7
	Polyglyceryl-3 oleate	Caprol® 3GO (ABITEC), Drewpol 3-1-O (Stepan)	6.5
30	Polyglyceryl-4 oleate	Nikkol Tetraglyn 1-O (Nikko)	5-7
	Polyglyceryl-4 stearate	Nikkol Tetraglyn 1-S (Nikko)	5-6
	Polyglyceryl-6 oleate	Drewpol 6-1-O (Stepan), Nikkol Hexaglyn 1-O (Nikko)	9

1	Polyglyceryl-10 laurate	Nikkol Decaglyn 1-L (Nikko)	15
	Polyglyceryl-10 oleate	Nikkol Decaglyn 1-O (Nikko)	14
	Polyglyceryl-10 stearate	Nikkol Decaglyn 1-S (Nikko)	12
5	Polyglyceryl-6 ricinoleate	Nikkol Hexaglyn PR-15 (Nikko)	>8
	Polyglyceryl-10 linoleate	Nikkol Decaglyn 1-LN (Nikko)	12
	Polyglyceryl-6 pentaoleate	Nikkol Hexaglyn 5-O (Nikko)	<10
	Polyglyceryl-3 dioleate	Cremophor GO32 (BASF)	<10
10	Polyglyceryl-3 distearate	Cremophor GS32 (BASF)	<10
	Polyglyceryl-4 pentaoleate	Nikkol Tetraglyn 5-O (Nikko)	<10
	Polyglyceryl-6 dioleate	Caprol® 6G20 (ABITEC); Hodag PGO-62 (Calgene), PLUROL OLEIQUE CC 497 (Gattefosse)	8.5
	Polyglyceryl-2 dioleate	Nikkol DGDO (Nikko)	7
15	Polyglyceryl-10 trioleate	Nikkol Decaglyn 3-O (Nikko)	7
	Polyglyceryl-10 pentaoleate	Nikkol Decaglyn 5-O (Nikko)	3.5
	Polyglyceryl-10 septaoleate	Nikkol Decaglyn 7-O (Nikko)	3
20	Polyglyceryl-10 tetraoleate	Caprol® 10G40 (ABITEC); Hodag PGO-62 (CALGENE), Drewpol 10-4-O (Stepan)	6.2
	Polyglyceryl-10 decaisostearate	Nikkol Decaglyn 10-IS (Nikko)	<10
	Polyglyceryl-10l decaoleate	Drewpol 10-10-O (Stepan), Caprol 10G100 (ABITEC), Nikkol Decaglyn 10-O	3.5
25	Polyglyceryl-10 mono, dioleate	Caprol® PGE 860 (ABITEC)	11
	Polyglyceryl polyricinoleate	Polymuls (Henkel)	3-20

30 1.7. Propylene Glycol Fatty Acid Esters

Esters of propylene glycol and fatty acids are suitable surfactants for use in the present invention. In this surfactant class, preferred hydrophobic surfactants include propylene glycol monolaurate (Lauroglycol FCC), propylene glycol ricinoleate (Propymuls),

1 propylene glycol monooleate (Myverol P-O6), propylene glycol dicaprylate/dicaprate (Captex® 200), and propylene glycol dioctanoate (Captex® 800). Examples of surfactants of this class are given in Table 7.

Table 7: Propylene Glycol Fatty Acid Esters

5	COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
	Propylene glycol monocaprylate	Capryol 90 (Gattefosse), Nikkol Sefsol 218 (Nikko)	<10
	Propylene glycol monolaurate	Lauroglycol 90 (Gattefosse), Lauroglycol FCC (Gattefosse)	<10
10	Propylene glycol oleate	Lutrol OP2000 (BASF)	<10
	Propylene glycol myristate	Mirpyl	<10
	Propylene glycol monostearate	ADM PGME-03 (ADM), LIPO PGMS (Lipo Chem.), Aldo® PGHMS (Lonza)	3-4
	Propylene glycol hydroxy stearate		<10
15	Propylene glycol ricinoleate	PROPYMULS (Henkel)	<10
	Propylene glycol isostearate		<10
	Propylene glycol monooleate	Myverol P-O6 (Eastman)	<10
20	Propylene glycol dicaprylate/dicaprate	Captex® 200 (ABITEC), Miglyol® 840 (Hüls), Neobee® M-20 (Stepan)	>6
	Propylene glycol dioctanoate	Captex® 800 (ABITEC)	>6
25	Propylene glycol caprylate/caprinate	LABRAFAC PG (Gattefosse)	>6
	Propylene glycol dilaurate		>6
	Propylene glycol distearate	Kessco® PGDS (Stepan)	>6
	Propylene glycol dicaprylate	Nikkol Sefsol 228 (Nikko)	>6
30	Propylene glycol dicaprate	Nikkol PDD (Nikko)	>6

1.8. Mixtures of Propylene Glycol Esters - Glycerol Esters

In general, mixtures of surfactants are also suitable for use in the present invention. In particular, mixtures of propylene glycol fatty acid esters and glycerol fatty acid esters are suitable and are commercially available. One preferred mixture is composed of the oleic acid esters of propylene glycol and glycerol (Arlacel 186). Examples of these surfactants are shown in Table 8.

Table 8: Glycerol/Propylene Glycol Fatty Acid Esters

COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
Oleic	ATMOS 300, ARLACEL 186 (ICI)	3-4
Stearic	ATMOS 150	3-4

1.9. Mono- and Diglycerides

A particularly important class of surfactants is the class of mono- and diglycerides. These surfactants are generally hydrophobic. Preferred hydrophobic surfactants in this class of compounds include glyceryl monooleate (Peceol), glyceryl ricinoleate, glyceryl laurate, glyceryl dilaurate (Capmul® GDL), glyceryl dioleate (Capmul® GDO), glyceryl mono/dioleate (Capmul® GMO-K), glyceryl caprylate/caprate (Capmul® MCM), caprylic acid mono/diglycerides (Imwitor® 988), and mono- and diacetylated monoglycerides (Myvacet® 9-45). Examples of these surfactants are given in Table 9.

Table 9: Mono- and Diglyceride Surfactants

COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
Monopalmitolein (C16:1)	(Larodan)	<10
Monoelaidin (C18:1)	(Larodan)	<10
Monocaproin (C6)	(Larodan)	<10
Monocaprylin	(Larodan)	<10
Monocaprin	(Larodan)	<10
Monolaurin	(Larodan)	<10
Glyceryl monomyristate (C14)	Nikkol MGM (Nikko)	3-4
Glyceryl monooleate (C18:1)	PECEOL (Gattefosse), Hodag GMO-D, Nikkol MGO (Nikko)	3-4
Glyceryl monooleate	RYLO series (Danisco), DIMODAN series (Danisco), EMULDAN (Danisco), ALDO® MO FG (Lonza),	3-4

1		EMULDAN (Danisco), ALDO® MO FG (Lonza), Kessco GMO (Stepan), MONOMULS® series (Henkel), TEGIN O, DREWMULSE GMO (Stepan), Atlas G-695 (ICI), GMOrphic 80 (Eastman), ADM DMG-40, 70, and 100 (ADM), Myverol (Eastman)	
5	Glycerol monooleate/linoleate	OLICINE (Gattefosse)	3-4
	Glycerol monolinoleate	Maisine (Gattefosse), MYVEROL 18-92, Myverol 18-06 (Eastman)	3-4
	Glyceryl ricinoleate	Softigen® 701 (Hüls), HODAG GMR-D (Calgene), ALDO® MR (Lonza)	6
10	Glyceryl monolaurate	ALDO® MLD (Lonza), Hodag GML (Calgene)	6.8
	Glycerol monopalmitate	Emalex GMS-P (Nihon)	4
	Glycerol monostearate	Capmul® GMS (ABITEC), Myvaplex (Eastman), IMWITOR® 191 (Hüls), CUTINA GMS, Aldo® MS (Lonza), Nikkol MGS series (Nikko)	5-9
15	Glyceryl mono-,dioleate	Capmul® GMO-K (ABITEC)	<10
	Glyceryl palmitic/stearic	CUTINA MD-A, ESTAGEL-G18	<10
	Glyceryl acetate	Lamegin® EE (Grünau GmbH)	<10
	Glyceryl laurate	Imwitor® 312 (Hüls), Monomuls® 90-45 (Grünau GmbH), Aldo® MLD (Lonza)	4
20	Glyceryl citrate/lactate/oleate/linoleate	Imwitor® 375 (Hüls)	<10
	Glyceryl caprylate	Imwitor® 308 (Hüls), Capmul® MCMC8 (ABITEC)	5-6
	Glyceryl caprylate/caprates	Capmul® MCM (ABITEC)	5-6
25	Caprylic acid mono,diglycerides	Imwitor® 988 (Hüls)	5-6
	Caprylic/capric glycerides	Imwitor® 742 (Hüls)	<10
	Mono-and diacetylated monoglycerides	Myvacet® 9-45, Myvacet® 9-40, Myvacet® 9-08 (Eastman), Lamegin® (Grünau)	3.8-4
30	Glyceryl monostearate	Aldo® MS, Arlacel 129 (ICI), LIPO GMS (Lipo Chem.), Imwitor® 191 (Hüls), Myvaplex (Eastman)	4.4
	Lactic acid esters of mono,diglycerides	LAMEGIN GLP (Henkel)	<10

1	Dicaproin (C6)	(Larodan)	<10
	Dicaprin (C10)	(Larodan)	<10
	Diocetanoïn (C8)	(Larodan)	<10
5	Dimyristin (C14)	(Larodan)	<10
	Dipalmitin (C16)	(Larodan)	<10
	Distearin	(Larodan)	<10
	Glyceryl dilaurate (C12)	Capmul® GDL (ABITEC)	3-4
10	Glyceryl dioleate	Capmul® GDO (ABITEC)	3-4
	Glycerol esters of fatty acids	GELUCIRE 39/01 (Gattefosse), GELUCIRE 43/01 (Gattefosse)	1
		GELUCIRE 37/06 (Gattefosse)	6
	Dipalmitolein (C16:1)	(Larodan)	<10
15	1,2 and 1,3-diolein (C18:1)	(Larodan)	<10
	Dielaidin (C18:1)	(Larodan)	<10
	Dilinolein (C18:2)	(Larodan)	<10

1.10. Sterol and Sterol Derivatives

20 Sterols and derivatives of sterols are suitable surfactants for use in the present invention. These surfactants can be hydrophilic or hydrophobic. Preferred derivatives include the polyethylene glycol derivatives. A preferred hydrophobic surfactant in this class is cholesterol. A preferred hydrophilic surfactant in this class is PEG-24 cholesterol ether (Solulan C-24). Examples of surfactants of this class are shown in Table 10.

25 Table 10: Sterol and Sterol Derivative Surfactants

	COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
	Cholesterol, sitosterol, lanosterol		<10
30	PEG-24 cholesterol ether	Solulan C-24 (Amerchol)	>10
	PEG-30 cholestanol	Nikkol DHC (Nikko)	>10
	Phytosterol	GENEROL series (Henkel)	<10
	PEG-25 phyto sterol	Nikkol BPSH-25 (Nikko)	>10

1	PEG-5 soya sterol	Nikkol BPS-5 (Nikko)	<10
	PEG-10 soya sterol	Nikkol BPS-10 (Nikko)	<10
	PEG-20 soya sterol	Nikkol BPS-20 (Nikko)	<10
5	PEG-30 soya sterol	Nikkol BPS-30 (Nikko)	>10

1.11. Polyethylene Glycol Sorbitan Fatty Acid Esters

A variety of PEG-sorbitan fatty acid esters are available and are suitable for use as surfactants in the present invention. In general, these surfactants are hydrophilic, although several hydrophobic surfactants of this class can be used. Among the PEG-sorbitan fatty acid esters, preferred hydrophilic surfactants include PEG-20 sorbitan monolaurate (Tween-20), PEG-20 sorbitan monopalmitate (Tween-40), PEG-20 sorbitan monostearate (Tween-60), and PEG-20 sorbitan monooleate (Tween-80). Examples of these surfactants are shown in Table 11.

15	Table 11: PEG-Sorbitan Fatty Acid Esters		
	COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
	PEG-10 sorbitan laurate	Liposorb L-10 (Lipo Chem.)	>10
	PEG-20 sorbitan monolaurate	Tween-20 (Atlas/ICI), Crillet 1 (Croda), DACOL MLS 20 (Condea)	17
20	PEG-4 sorbitan monolaurate	Tween-21 (Atlas/ICI), Crillet 11 (Croda)	13
	PEG-80 sorbitan monolaurate	Hodag PSML-80 (Calgene); T-Maz 28	>10
25	PEG-6 sorbitan monolaurate	Nikkol GL-1 (Nikko)	16
	PEG-20 sorbitan monopalmitate	Tween-40 (Atlas/ICI), Crillet 2 (Croda)	16
	PEG-20 sorbitan monostearate	Tween-60 (Atlas/ICI), Crillet 3 (Croda)	15
30	PEG-4 sorbitan monostearate	Tween-61 (Atlas/ICI), Crillet 31 (Croda)	9.6
	PEG-8 sorbitan monostearate	DACOL MSS (Condea)	>10

1	PEG-6 sorbitan monostearate	Nikkol TS106 (Nikko)	11
	PEG-20 sorbitan tristearate	Tween-65 (Atlas/ICI), Crillet 35 (Croda)	11
5	PEG-6 sorbitan tetrastearate	Nikkol GS-6 (Nikko)	3
	PEG-60 sorbitan tetrastearate	Nikkol GS-460 (Nikko)	13
	PEG-5 sorbitan monooleate	Tween-81 (Atlas/ICI), Crillet 41 (Croda)	10
	PEG-6 sorbitan monooleate	Nikkol TO-106 (Nikko)	10
10	PEG-20 sorbitan monooleate	Tween-80 (Atlas/ICI), Crillet 4 (Croda)	15
	PEG-40 sorbitan oleate	Emalex ET 8040 (Nihon Emulsion)	18
	PEG-20 sorbitan trioleate	Tween-85 (Atlas/ICI), Crillet 45 (Croda)	11
15	PEG-6 sorbitan tetraoleate	Nikkol GO-4 (Nikko)	8.5
	PEG-30 sorbitan tetraoleate	Nikkol GO-430 (Nikko)	12
	PEG-40 sorbitan tetraoleate	Nikkol GO-440 (Nikko)	13
	PEG-20 sorbitan monoisostearate	Tween-120 (Atlas/ICI), Crillet 6 (Croda)	>10
20	PEG sorbitol hexaoleate	Atlas G-1086 (ICI)	10
	PEG-6 sorbitol hexastearate	Nikkol GS-6 (Nikko)	3

1.12. Polyethylene Glycol Alkyl Ethers

25 Ethers of polyethylene glycol and alkyl alcohols are suitable surfactants for use in the present invention. Preferred hydrophobic ethers include PEG-3 oleyl ether (Volpo 3) and PEG-4 lauryl ether (Brij 30). Examples of these surfactants are shown in Table 12.

Table 12: Polyethylene Glycol Alkyl Ethers

30	COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
	PEG-2 oleyl ether,oleth-2	Brij 92/93 (Atlas/ICI)	4.9
	PEG-3 oleyl ether,oleth-3	Volpo 3 (Croda)	<10
	PEG-5 oleyl ether,oleth-5	Volpo 5 (Croda)	<10

1	PEG-10 oleyl ether, oleth-10	Volpo 10 (Croda), Brij 96/97 (Atlas/ICI)	12
	PEG-20 oleyl ether, oleth-20	Volpo 20 (Croda), Brij 98/99 (Atlas/ICI)	15
5	PEG-4 lauryl ether, laureth-4	Brij 30 (Atlas/ICI)	9.7
	PEG-9 lauryl ether		>10
	PEG-23 lauryl ether, laureth-23	Brij 35 (Atlas/ICI)	17
10	PEG-2 cetyl ether	Brij 52 (ICI)	5.3
	PEG-10 cetyl ether	Brij 56 (ICI)	13
	PEG-20 cetyl ether	Brij 58 (ICI)	16
	PEG-2 stearyl ether	Brij 72 (ICI)	4.9
15	PEG-10 stearyl ether	Brij 76 (ICI)	12
	PEG-20 stearyl ether	Brij 78 (ICI)	15
	PEG-100 stearyl ether	Brij 700 (ICI)	>10

1.13. Sugar Esters

20 Esters of sugars are suitable surfactants for use in the present invention. Preferred hydrophilic surfactants in this class include sucrose monopalmitate and sucrose monolaurate. Examples of such surfactants are shown in Table 13.

Table 13: Sugar Ester Surfactants

	COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
25	Sucrose distearate	SUCRO ESTER 7 (Gattefosse), Crodesta F-10 (Croda)	3
	Sucrose distearate/monostearate	SUCRO ESTER 11 (Gattefosse), Crodesta F-110 (Croda)	12
	Sucrose dipalmitate		7.4
30	Sucrose monostearate	Crodesta F-160 (Croda)	15
	Sucrose monopalmitate	SUCRO ESTER 15 (Gattefosse)	>10
	Sucrose monolaurate	Saccharose monolaurate 1695 (Mitsubishi-Kasei)	15

1.14. Polyethylene Glycol Alkyl Phenols

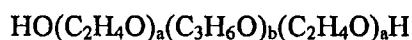
Several hydrophilic PEG-alkyl phenol surfactants are available, and are suitable for use in the present invention. Examples of these surfactants are shown in Table 14.

Table 14: Polyethylene Glycol Alkyl Phenol Surfactants

COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
PEG-10-100 nonyl phenol	Triton X series (Rohm & Haas), Igepal CA series (GAF, USA), Antarox CA series (GAF, UK)	>10
PEG-15-100 octyl phenol ether	Triton N-series (Rohm & Haas), Igepal CO series (GAF, USA), Antarox CO series (GAF, UK)	>10

1.15. Polyoxyethylene-Polyoxypropylene Block Copolymers

The POE-POP block copolymers are a unique class of polymeric surfactants. The unique structure of the surfactants, with hydrophilic POE and hydrophobic POP moieties in well-defined ratios and positions, provides a wide variety of surfactants suitable for use in the present invention. These surfactants are available under various trade names, including Synperonic PE series (ICI); Pluronic® series (BASF), Emkalyx, Lutrol (BASF), Supronic, Monolan, Pluracare, and Plurodac. The generic term for these polymers is "poloxamer" (CAS 9003-11-6). These polymers have the formula:



where "a" and "b" denote the number of polyoxyethylene and polyoxypropylene units, respectively.

Preferred hydrophilic surfactants of this class include Poloxamers 108, 188, 217, 238, 288, 338, and 407. Preferred hydrophobic surfactants in this class include Poloxamers 124, 182, 183, 212, 331, and 335.

Examples of suitable surfactants of this class are shown in Table 15. Since the compounds are widely available, commercial sources are not listed in the Table. The compounds are listed by generic name, with the corresponding "a" and "b" values.

Table 15: POE-POP Block Copolymers

COMPOUND	a, b values in $\text{HO}(\text{C}_2\text{H}_4\text{O})_a(\text{C}_3\text{H}_6\text{O})_b(\text{C}_2\text{H}_4\text{O})_a\text{H}$	HLB
Poloxamer 105	a = 11 b = 16	8

1	Poloxamer 108	a = 46 b = 16	>10
	Poloxamer 122	a = 5 b = 21	3
	Poloxamer 123	a = 7 b = 21	7
5	Poloxamer 124	a = 11 b = 21	>7
	Poloxamer 181	a = 3 b = 30	
	Poloxamer 182	a = 8 b = 30	2
	Poloxamer 183	a = 10 b = 30	
	Poloxamer 184	a = 13 b = 30	
10	Poloxamer 185	a = 19 b = 30	
	Poloxamer 188	a = 75 b = 30	29
	Poloxamer 212	a = 8 b = 35	
	Poloxamer 215	a = 24 b = 35	
	Poloxamer 217	a = 52 b = 35	
15	Poloxamer 231	a = 16 b = 39	
	Poloxamer 234	a = 22 b = 39	
	Poloxamer 235	a = 27 b = 39	
	Poloxamer 237	a = 62 b = 39	24
	Poloxamer 238	a = 97 b = 39	
20	Poloxamer 282	a = 10 b = 47	
	Poloxamer 284	a = 21 b = 47	
	Poloxamer 288	a = 122 b = 47	>10
	Poloxamer 331	a = 7 b = 54	0.5
25	Poloxamer 333	a = 20 b = 54	
	Poloxamer 334	a = 31 b = 54	
	Poloxamer 335	a = 38 b = 54	
	Poloxamer 338	a = 128 b = 54	
	Poloxamer 401	a = 6 b = 67	
30	Poloxamer 402	a = 13 b = 67	
	Poloxamer 403	a = 21 b = 67	
	Poloxamer 407	a = 98 b = 67	

1.16. Sorbitan Fatty Acid Esters

Sorbitan esters of fatty acids are suitable surfactants for use in the present invention. Among these esters, preferred hydrophobic surfactants include sorbitan monolaurate (Arlacel 20), sorbitan monopalmitate (Span-40), sorbitan monooleate (Span-80), sorbitan monostearate, and sorbitan tristearate. Examples of these surfactants are shown in Table 16.

Table 16: Sorbitan Fatty Acid Ester Surfactants

COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
Sorbitan monolaurate	Span-20 (Atlas/ICI), Crill 1 (Croda), Arlacel 20 (ICI)	8.6
Sorbitan monopalmitate	Span-40 (Atlas/ICI), Crill 2 (Croda), Nikkol SP-10 (Nikko)	6.7
Sorbitan monooleate	Span-80 (Atlas/ICI), Crill 4 (Croda), Crill 50 (Croda)	4.3
Sorbitan monostearate	Span-60 (Atlas/ICI), Crill 3 (Croda), Nikkol SS-10 (Nikko)	4.7
Sorbitan trioleate	Span-85 (Atlas/ICI), Crill 45 (Croda), Nikkol SO-30 (Nikko)	4.3
Sorbitan sesquioleate	Arlacel-C (ICI), Crill 43 (Croda), Nikkol SO-15 (Nikko)	3.7
Sorbitan tristearate	Span-65 (Atlas/ICI) Crill 35 (Croda), Nikkol SS-30 (Nikko)	2.1
Sorbitan monoisostearate	Crill 6 (Croda), Nikkol SI-10 (Nikko)	4.7
Sorbitan sesquisteate	Nikkol SS-15 (Nikko)	4.2

1.17. Lower Alcohol Fatty Acid Esters

Esters of lower alcohols (C_2 to C_4) and fatty acids (C_8 to C_{18}) are suitable surfactants for use in the present invention. Among these esters, preferred hydrophobic surfactants include ethyl oleate (Crodamol EO), isopropyl myristate (Crodamol IPM), and isopropyl palmitate (Crodamol IPP). Examples of these surfactants are shown in Table 17.

Table 17: Lower Alcohol Fatty Acid Ester Surfactants

COMPOUND	COMMERCIAL PRODUCT (Supplier)	HLB
Ethyl oleate	Crodamol EO (Croda), Nikkol EEO (Nikko)	<10
Isopropyl myristate	Crodamol IPM (Croda)	<10
Isopropyl palmitate	Crodamol IPP (Croda)	<10

1	Ethyl linoleate	Nikkol VF-E (Nikko)	<10
	Isopropyl linoleate	Nikkol VF-IP (Nikko)	<10

5 1.18. Ionic Surfactants

Ionic surfactants, including cationic, anionic and zwitterionic surfactants, are suitable hydrophilic surfactants for use in the present invention. Preferred anionic surfactants include fatty acid salts and bile salts. Specifically, preferred ionic surfactants include sodium oleate, sodium lauryl sulfate, sodium lauryl sarcosinate, sodium dioctyl sulfosuccinate, sodium cholate, and sodium taurocholate. Examples of such surfactants are shown in Table 18 below. For simplicity, typical counterions are shown in the entries in the Table. It will be appreciated by one skilled in the art, however, that any bioacceptable counterion may be used. For example, although the fatty acids are shown as sodium salts, other cation counterions can also be used, such as alkali metal cations or ammonium. Unlike typical non-ionic surfactants, these ionic surfactants are generally available as pure compounds, rather than commercial (proprietary) mixtures. Because these compounds are readily available from a variety of commercial suppliers, such as Aldrich, Sigma, and the like, commercial sources are not generally listed in the Table.

Table 18: Ionic Surfactants

20	COMPOUND	HLB
	FATTY ACID SALTS	>10
	Sodium caproate	
	Sodium caprylate	
	Sodium caprate	
25	Sodium laurate	
	Sodium myristate	
	Sodium myristolate	
	Sodium palmitate	
	Sodium palmitoleate	
	Sodium oleate	18
30	Sodium ricinoleate	
	Sodium linoleate	
	Sodium linolenate	
	Sodium stearate	

1	Sodium lauryl sulfate (dodecyl)	40
	Sodium tetradecyl sulfate	
	Sodium lauryl sarcosinate	
	Sodium dioctyl sulfosuccinate [sodium docusate (Cytec)]	
5	BILE SALTS	>10
	Sodium cholate	
	Sodium taurocholate	
	Sodium glycocholate	
	Sodium deoxycholate	
	Sodium taurodeoxycholate	
10	Sodium glycodeoxycholate	
	Sodium ursodeoxycholate	
	Sodium chenodeoxycholate	
	Sodium taurochenodeoxycholate	
	Sodium glyco cheno deoxycholate	
	Sodium cholylsarcosinate	
15	Sodium N-methyl taurocholate	
	PHOSPHOLIPIDS	
	Egg/Soy lecithin [Epikuron™ (Lucas Meyer), Ovothin™ (Lucas Meyer)]	
	Lyso egg/soy lecithin	
20	Hydroxylated lecithin	
	Lysophosphatidylcholine	
	Cardiolipin	
	Sphingomyelin	
	Phosphatidylcholine	
25	Phosphatidyl ethanolamine	
	Phosphatidic acid	
	Phosphatidyl glycerol	
	Phosphatidyl serine	
	PHOSPHORIC ACID ESTERS	
30	Diethanolammonium polyoxyethylene-10 oleyl ether phosphate	
	Esterification products of fatty alcohols or fatty alcohol ethoxylates with phosphoric acid or anhydride	

1

CARBOXYLATES

Ether carboxylates (by oxidation of terminal OH group of fatty alcohol ethoxylates)

Succinylated monoglycerides [LAMEGIN ZE (Henkel)]

5

Sodium stearyl fumarate

Stearoyl propylene glycol hydrogen succinate

Mono/diacetylated tartaric acid esters of mono- and diglycerides

Citric acid esters of mono-, diglycerides

Glyceryl-lacto esters of fatty acids (CFR ref. 172.852)

Acyl lactylates:

10

lactic esters of fatty acids

calcium/sodium stearyl-2-lactylate

calcium/sodium stearyl lactylate

Alginate salts

Propylene glycol alginate

SULFATES AND SULFONATES

Ethoxylated alkyl sulfates

15

Alkyl benzene sulfones

α -olefin sulfonates

Acyl isethionates

Acyl taurates

Alkyl glyceryl ether sulfonates

Octyl sulfosuccinate disodium

20

Disodium undecylenamideo-MEA-sulfosuccinate

CATIONIC Surfactants

>10

Hexadecyl triammonium bromide

Decyl trimethyl ammonium bromide

Cetyl trimethyl ammonium bromide

25

Dodecyl ammonium chloride

Alkyl benzyldimethylammonium salts

Diisobutyl phenoxyethoxydimethyl benzylammonium salts

Alkylpyridinium salts

Betaines (trialkylglycine):

Lauryl betaine (N-lauryl,N,N-dimethylglycine)

30

Ethoxylated amines:

Polyoxyethylene-15 coconut amine

1 1.19 Surfactant Concentrations

 The hydrophilic and hydrophobic surfactants are present in the pharmaceutical compositions of the present invention in amounts such that upon dilution with an aqueous solution, the carrier forms a clear, aqueous dispersion of the hydrophilic and hydrophobic
5 surfactants, containing the hydrophobic therapeutic agent. The relative amounts of hydrophilic and hydrophobic surfactants are readily determined by observing the properties of the resultant dispersion; *i.e.*, when the relative amounts of the hydrophobic and hydrophilic surfactants are within a suitable range, the resultant aqueous dispersion is optically clear. When the relative amount of hydrophobic surfactant is too great, the resulting dispersion is
10 visibly "cloudy", resembling a conventional emulsion or multiple phase system. Although a visibly cloudy solution may be potentially useful for some applications, such a system would suffer from many of the same disadvantages as conventional prior art formulations, as described above.

 A convenient method of determining the appropriate relative concentrations for any
15 hydrophilic surfactant - hydrophobic surfactant pair is as follows. A convenient working amount of a hydrophilic surfactant is provided, and a known amount of a hydrophobic surfactant is added. The surfactants are stirred to form a homogeneous mixture, with the aid of gentle heating if desired. The resulting mixture is diluted with purified water to prepare an aqueous dispersion. Any dilution amount can be chosen, but convenient dilutions are those
20 within the range expected *in vivo*, about a 10 to 250-fold dilution. The aqueous dispersion is then assessed qualitatively for optical clarity. The procedure can be repeated with incremental variations in the relative amount of hydrophobic surfactant added, to determine the maximum relative amount of hydrophobic surfactant that can be present for a given surfactant pair.

25 Alternatively, the optical clarity of the aqueous dispersion can be measured using standard quantitative techniques for turbidity assessment. One convenient procedure to measure turbidity is to measure the amount of light of a given wavelength transmitted by the solution, using, for example, a UV-visible spectrophotometer. Using this measure, optical clarity corresponds to high transmittance, since cloudier solutions will scatter more of the
30 incident radiation, resulting in lower transmittance measurements. If this procedure is used, care should be taken to insure that the surfactant mixture does not itself absorb light of the chosen wavelength, as any true absorbance necessarily reduces the amount of transmitted light and falsely increases the quantitative turbidity value. In the absence of chromophores at

1 the chosen wavelength, suitable dispersions at a dilution of 10X should have an apparent
absorbance of less than about 0.3, preferably less than about 0.2, and more preferably less
than about 0.1. At a dilution of 100X, suitable dispersions should have an apparent
absorbance of less than about 0.1, preferably less than about 0.05, and more preferably less
5 than about 0.01.

A third method of determining optical clarity and carrier diffusivity through the
aqueous boundary layer is to quantitatively measure the size of the particles of which the
dispersion is composed. These measurements can be performed on commercially available
particle size analyzers, such as, for example, a Nicomp particle size analyzer available from
10 Particle Size Systems, Inc., of Santa Barbara, CA. Using this measure, clear aqueous
dispersions according to the present invention have average particle sizes much smaller than
the wavelength of visible light, whereas dispersions containing excessive relative amounts of
the hydrophobic surfactant have more complex particle size distributions, with much greater
average particle sizes. It is desirable that the average particle size be less than about 100 nm,
15 preferably less than about 50 nm, more preferably less than about 30 nm, and still more
preferably less than about 20 nm. It is also preferred that the particle size distribution be
mono-modal. As is shown in more detail in the Examples herein, dispersions having an
undesirably large relative amount of hydrophobic surfactant typically display bimodal
particle size distributions, such distributions having a small particle size component, typically
20 less than about 30 nm, and a large particle size component, typically on the order of 100 nm
or more. It should be emphasized that these particle sizes are appropriate for the carrier
particles in aqueous solution, in the absence of a hydrophobic therapeutic agent. It is
expected that the presence of the hydrophobic therapeutic agent may result in an increase in
the average particle size.

25 Other methods of determining optical clarity or particle size can be used as desired.
Such methods are well known to those skilled in the art.

It should be emphasized that any or all of the available methods may be used to
ensure that the resulting aqueous dispersions possess the requisite optical clarity. For
convenience, however, the present inventors prefer to use the simple qualitative procedure;
30 *i.e.*, simple visible observation. However, in order to more fully illustrate the practice of the
present invention, all three of the above measures are used to assess the dispersion clarity in
the Examples herein.

1 Although it should be understood that any aqueous dispersion having the properties
described above is within the scope of the present invention regardless of the specific relative
amounts of hydrophobic and hydrophilic surfactants, it is expected that the amount of
hydrophobic surfactant will generally be less than about 200% by weight, based on the
5 amount of hydrophilic surfactant, and more specifically, in the range of about 1% to 200%.
Further, based on observations reported in the Examples herein, it is expected that the amount
of hydrophobic surfactant will generally be less than about 100%, and more specifically in
the range of about 5% to about 100% by weight, or about 10% to about 100% by weight,
based on the amount of hydrophilic surfactant. For some particular surfactant combinations,
10 cloudy solutions result when the amount of hydrophobic surfactant is greater than about 60%
by weight, based on the amount of hydrophilic surfactant. A preferred range for these
surfactants is about 1% to about 60%, preferably about 5% to about 60%, and more
preferably about 10% to about 60%. Addition of optional excipients as described below can
further increase the maximum relative amount of hydrophobic surfactant that can be used.

15 Other considerations well known to those skilled in the art will further inform the
choice of specific proportions of hydrophobic and hydrophilic surfactants. These
considerations include the degree of bioacceptability of the surfactants, and the desired
dosage of hydrophobic therapeutic agent to be provided. In some cases, the amount of
hydrophobic surfactant actually used in a pharmaceutical composition according to the
20 present invention will be less than the maximum that can be used, and it should be apparent
that such compositions are also within the scope of the present invention.

2. Hydrophobic Therapeutic Agents

Hydrophobic therapeutic agents suitable for use in the pharmaceutical compositions
of the present invention are not particularly limited, as the carrier is surprisingly capable of
25 solubilizing and delivering a wide variety of hydrophobic therapeutic agents. Hydrophobic
therapeutic agents are compounds with little or no water solubility. Intrinsic water
solubilities (*i.e.*, water solubility of the unionized form) for hydrophobic therapeutic agents
usable in the present invention are less than about 1% by weight, and typically less than about
0.1% or 0.01% by weight. Such therapeutic agents can be any agents having therapeutic or
30 other value when administered to an animal, particularly to a mammal, such as drugs,
nutrients, and cosmetics (cosmeceuticals). It should be understood that while the invention is
described with particular reference to its value in the form of aqueous dispersions, the
invention is not so limited. Thus, hydrophobic drugs, nutrients or cosmetics which derive

1 their therapeutic or other value from, for example, topical or transdermal administration, are still considered to be suitable for use in the present invention.

Specific non-limiting examples of hydrophobic therapeutic agents that can be used in the pharmaceutical compositions of the present invention include the following representative
5 compounds, as well as their pharmaceutically acceptable salts, isomers, esters, ethers and other derivatives:

analgesics and anti-inflammatory agents, such as aloxiprin, auranofin, azapropazone, benorylate, capsaicin, celecoxib, diclofenac, diflunisal, etodolac, fenbufen, fenoprofen calcium, flurbiprofen, ibuprofen, indomethacin, ketoprofen, ketorolac, leflunomide,
10 meclofenamic acid, mefenamic acid, nabumetone, naproxen, oxaprozin, oxyphenbutazone, phenylbutazone, piroxicam, refocoxib, sulindac, tetrahydrocannabinol, tramadol and tromethamine;

anthelmintics, such as albendazole, bethovenium hydroxynaphthoate, cambendazole, dichlorophen, ivermectin, mebendazole, oxamniquine, oxfendazole, oxantel embonate,
15 praziquantel, pyrantel embonate and thiabendazole;

anti-arrhythmic agents, such as amiodarone HCl, disopyramide, flecainide acetate and quinidine sulfate;

anti-asthma agents, such as zileuton, zafirlukast, terbutaline sulfate, montelukast, and albuterol;

20 anti-bacterial agents, such as alatrofloxacin, azithromycin, baclofen, benethamine penicillin, cinoxacin, ciprofloxacin HCl, clarithromycin, clofazimine, cloxacillin, demeclocycline, dirithromycin, doxycycline, erythromycin, ethionamide, furazolidone, grepafloxacin, imipenem, levofloxacin, loxofloxacin, moxifloxacin HCl, nalidixic acid, nitrofurantoin, norfloxacin, ofloxacin, rifampicin, rifabutine, rifapentine, sparfloxacin,
25 spiramycin, sulphabenzamide, sulphadoxine, sulphamerazine, sulphacetamide, sulphadiazine, sulphafurazole, sulphamethoxazole, sulphapyridine, tetracycline, trimethoprim, trovafloxacin, and vancomycin;

anti-viral agents, such as abacavir, amprenavir, delavirdine, efavirenz, indinavir, lamivudine, nelfinavir, nevirapine, ritonavir, saquinavir, and stavudine;

30 anti-coagulants, such as cilostazol, clopidogrel, dicoumarol, dipyridamole, nicoumalone, oprelvekin, phenindione, ticlidopine, and tirofiban;

anti-depressants, such as amoxapine, bupropion, citalopram, clomipramine, fluoxetine HCl, maprotiline HCl, mianserin HCl, nortriptyline HCl, paroxetine HCl, sertraline HCl,

- 1 trazodone HCl, trimipramine maleate, and venlafaxine HCl;
 anti-diabetics, such as acetohexamide, chlorpropamide, glibenclamide, gliclazide, glipizide, glymepride, miglitol, pioglitazone, repaglinide, rosiglitazone, tolazamide, tolbutamide and troglitazone;
- 5 anti-epileptics, such as beclamide, carbamazepine, clonazepam, ethosin, felbamate, fosphenytoin sodium, lamotrigine, methoin, methsuximide, methylphenobarbitone, oxcarbazepine, paramethadione, phenacemide, phenobarbitone, phenytoin, phensuximide, primidone, sulthiame, tiagabine HCl, topiramate, valproic acid, and vigabatrin;
 anti-fungal agents, such as amphotericin, butenafine HCl, butoconazole nitrate,
- 10 clotrimazole, econazole nitrate, fluconazole, flucytosine, griseofulvin, itraconazole, ketoconazole, miconazole, natamycin, nystatin, sulconazole nitrate, oxiconazole, terbinafine HCl, terconazole, tioconazole and undecenoic acid;
 anti-gout agents, such as allopurinol, probenecid and sulphin-pyrazone;
 anti-hypertensive agents, such as amlodipine, benidipine, benazepril, candesartan,
- 15 captopril, darodipine, diltiazem HCl, diazoxide, doxazosin HCl, elanapril, eprosartan losartan, mesylate, felodipine, fenoldopam, fosinopril, guanabenz acetate, irbesartan, isradipine, lisinopril, minoxidil, nifedipine HCl, nifedipine, nimodipine, nisoldipine, phenoxybenzamine HCl, prazosin HCl, quinapril, reserpine, terazosin HCl, telmisartan, and valsartan;
- 20 anti-malarials, such as amodiaquine, chloroquine, chlorproguanil HCl, halofantrine HCl, mefloquine HCl, proguanil HCl, pyrimethamine and quinine sulfate;
 anti-migraine agents, such as dihydroergotamine mesylate, ergotamine tartrate, frovatriptan, methysergide maleate, naratriptan HCl, pizotifen maleate, rizatriptan benzoate, sumatriptan succinate, and zolmitriptan;
- 25 anti-muscarinic agents, such as atropine, benzhexol HCl, biperiden, ethopropazine HCl, hyoscyamine, mepenzolate bromide, oxyphencylamine HCl and tropicamide;
 anti-neoplastic agents and immunosuppressants, such as aminoglutethimide, amsacrine, azathioprine, bicalutamide, bisanthrene, busulphan, camptothecin, capecitabine, chlorambucil, cyclosporin, dacarbazine, ellipticine, estramustine, etoposide, irinotecan,
- 30 lomustine, melphalan, mercaptopurine, methotrexate, mitomycin, mitotane, mitoxantrone, mofetil, mycophenolate, nilutamide, paclitaxel, procarbazine HCl, sirolimus, tacrolimus, tamoxifen citrate, teniposide, testolactone, topotecan HCl, and toremifene citrate;
 anti-protozoal agents, such as atovaquone, benznidazole, clioquinol, decoquinol,

1 diiodohydroxyquinoline, diloxanide furoate, dinitolmide, furzolidone, metronidazole, nimorazole, nitrofurazone, ornidazole and tinidazole;

anti-thyroid agents, such as carbimazole, paricalcitol, and propylthiouracil;

anti-tussives, such as benzonatate;

5 anxiolytic, sedatives, hypnotics and neuroleptics, such as alprazolam, amylobarbitone, barbitone, bentazepam, bromazepam, bromperidol, brotizolam, butobarbitone, carbromal, chlordiazepoxide, chlormethiazole, chlorpromazine, chlorprothiocene, clonazepam, clobazam, clotiazepam, clozapine, diazepam, droperidol, ethinamate, flunarisone, flunitrazepam, fluopromazine, flupenthixol decanoate, fluphenazine decanoate, flurazepam, 10 gabapentin, haloperidol, lorazepam, lormetazepam, medazepam, meprobamate, mesoridazine, methaqualone, methyl phenidate, midazolam, molindone, nitrazepam, olanzapine, oxazepam, pentobarbitone, perphenazine pimozide, prochlorperazine, pseudoephedrine, quetiapine, risperidone, sertindole, sulpiride, temazepam, thioridazine, triazolam, zolpidem, and zopiclone;

15 β -Blockers, such as acebutolol, alprenolol, atenolol, labetalol, metoprolol, nadolol, oxprenolol, pindolol and propranolol;

cardiac inotropic agents, such as amrinone, digitoxin, digoxin, enoximone, lanatoside C and medigoxin;

20 corticosteroids, such as beclomethasone, betamethasone, budesonide, cortisone acetate, desoxymethasone, dexamethasone, fludrocortisone acetate, flunisolide, flucortolone, fluticasone propionate, hydrocortisone, methylprednisolone, prednisolone, prednisone and triamcinolone;

25 diuretics, such as acetazolamide, amiloride, bendrofluazide, bumetanide, chlorothiazide, chlorthalidone, ethacrynic acid, frusemide, metolazone, spironolactone and triamterene.

anti-parkinsonian agents, such as bromocriptine mesylate, lysuride maleate, pramipexole, robinirole HCl, and tolcapone;

30 gastro-intestinal agents, such as bisacodyl, cimetidine, cisapride, diphenoxylate HCl, domperidone, famotidine, lansoprazole, loperamide, mesalazine, nizatidine, omeprazole, ondansetron HCl, rabeprazole sodium, ranitidine HCl and sulphasalazine;

histamine H₁-receptor antagonists, such as acrivastine, astemizole, chlophenisamine, cinnarizine, citirizine, clemastine fumarate, cyclizine, cyproheptadine HCl, dexchlorpheniramine, dimenhydrinate, fexofenadine, flunarizine HCl, loratadine, meclozine

1 HCl, oxatomide, and terenadine;

keratolytics, such as acutretin, calciprotiene, calcifediol, calcitriol, cholecalciferol, ergocalciferol, etretinate, retinoids, targretin, and tazarotene;

5 lipid regulating agents, such as atorvastatin, bezafibrate, cerivistatin, clinofibrate, clofibrate, fenofibrate, fluvastatin, gemfibrozil, pravastatin, probucol, and simvastatin;

muscle relaxants, such as dantrolene sodium and tizanidine HCl;

nitrates and other anti-anginal agents, such as amyl nitrate, glyceryl trinitrate, isosorbide dinitrate, isosorbide mononitrate and pentaerythritol tetranitrate;

10 nutritional agents, such as calcitriol, carotenes, dihydrotachysterol, essential fatty acids, non-essential fatty acids, phytonadione, vitamin A, vitamin B₂, vitamin D, vitamin E and vitamin K.

opioid analgesics, such as codeine, dextropropoxyphene, diamorphine, dihydrocodeine, fentanyl, meptazinol, methadone, morphine, nalbuphine and pentazocine;

15 sex hormones, such as clomiphene citrate, cortisone acetate, danazol, dihydro epiandrosterone, ethinyloestradiol, finasteride, fludrocortisone, fluoxymesterone, medroxyprogesterone acetate, megestrol acetate, mestranol, methyltestosterone, norethisterone, norgestrel, oestradiol, conjugated estrogens, progesterone, rimexolone, stanozolol, stibioestrol, testosterone and tibolone;

20 stimulants, such as amphetamine, dexamphetamine, dexfenfluramine, fenfluramine and mazindol;

and others, such as becaplermin, donepezil HCl, L-thyroxine, methoxsalen, nerteporfin, physostigmine, pyridostigmine, raloxifene HCl, sibutramine HCl, sildenafil citrate, tacrine, tamsulosin HCl, and tolterodine.

25 Preferred hydrophobic therapeutic agents include sildenafil citrate, amlodipine, tramadol, celecoxib, refocoxib, oxaprozin, nabumetone, ibuprofen, terbenafine, itraconazole, zileuton, zafirlukast, cisapride, fenofibrate, tizanidine, nizatidine, fexofenadine, loratadine, famotidine, paricalcitol, atovaquone, nabumetone, tetrahydrocannabinol, megestrol acetate, repaglinide, progesterone, rimexolone, cyclosporine, tacrolimus, sirolimus, teniposide, paclitaxel, pseudo-ephedrine, troglitazone, rosiglitazone, finasteride, vitamin A, vitamin D, 30 vitamin E, and pharmaceutically acceptable salts, isomers and derivatives thereof. Particularly preferred hydrophobic therapeutic agents are progesterone and cyclosporin.

It should be appreciated that this listing of hydrophobic therapeutic agents and their therapeutic classes is merely illustrative. Indeed, a particular feature, and surprising

1 advantage, of the compositions of the present invention is the ability of the present compositions to solubilize and deliver a broad range of hydrophobic therapeutic agents, regardless of functional class. Of course, mixtures of hydrophobic therapeutic agents may also be used where desired.

5 3. Solubilizers

If desired, the pharmaceutical compositions of the present invention can optionally include additional compounds to enhance the solubility of the hydrophobic therapeutic agent in the carrier system. Examples of such compounds, referred to as "solubilizers", include:

10 alcohols and polyols, such as ethanol, isopropanol, butanol, benzyl alcohol, ethylene glycol, propylene glycol, butanediols and isomers thereof, glycerol, pentaerythritol, sorbitol, mannitol, transcitol, dimethyl isosorbide, polyethylene glycol, polypropylene glycol, polyvinylalcohol, hydroxypropyl methylcellulose and other cellulose derivatives, cyclodextrins and cyclodextrin derivatives;

15 ethers of polyethylene glycols having an average molecular weight of about 200 to about 6000, such as tetrahydrofurfuryl alcohol PEG ether (glycofurol, available commercially from BASF under the trade name Tetraglycol) or methoxy PEG (Union Carbide);

amides, such as 2-pyrrolidone, 2-piperidone, ϵ -caprolactam, N-alkylpyrrolidone, N-hydroxyalkylpyrrolidone, N-alkylpiperidone, N-alkylcaprolactam, dimethylacetamide, and polyvinylpyrrolidone;

20 esters, such as ethyl propionate, tributylcitrate, acetyl triethylcitrate, acetyl tributyl citrate, triethylcitrate, ethyl oleate, ethyl caprylate, ethyl butyrate, triacetin, propylene glycol monoacetate, propylene glycol diacetate, ϵ -caprolactone and isomers thereof, δ -valerolactone and isomers thereof, β -butyrolactone and isomers thereof;

25 and other solubilizers known in the art, such as dimethyl acetamide, dimethyl isosorbide (Arlasolve DMI (ICI)), N-methyl pyrrolidones (Pharmasolve (ISP)), monooctanoin, diethylene glycol monoethyl ether (available from Gattefosse under the trade name Transcutol), and water.

Mixtures of solubilizers are also within the scope of the invention. Except as indicated, these compounds are readily available from standard commercial sources.

30 Preferred solubilizers include triacetin, triethylcitrate, ethyl oleate, ethyl caprylate, dimethylacetamide, N-methylpyrrolidone, N-hydroxyethylpyrrolidone, polyvinylpyrrolidone, hydroxypropyl methylcellulose, hydroxypropyl cyclodextrins, ethanol, polyethylene glycol

1 200-600, glycofurol, transcitol, propylene glycol, and dimethyl isosorbide. Particularly preferred solubilizers include sorbitol, glycerol, triacetin, ethyl alcohol, PEG-400, glycofurol and propylene glycol.

5 The amount of solubilizer that can be included in compositions of the present invention is not particularly limited. Of course, when such compositions are ultimately administered to a patient, the amount of a given solubilizer is limited to a bioacceptable amount, which is readily determined by one of skill in the art. In some circumstances, it may be advantageous to include amounts of solubilizers far in excess of bioacceptable amounts in order to maximize the concentration of hydrophobic therapeutic agent, with excess solubilizer removed prior to providing the composition to a patient using conventional techniques, such as distillation or evaporation. Thus, if present, the solubilizer can be in a concentration of 10 50%, 100%, 200%, or up to about 400% by weight, based on the amount of surfactant. If desired, very small amounts of solubilizers may also be used, such as 25%, 10%, 5%, 1% or even less. Typically, the solubilizer will be present in an amount of about 1% to about 100%, 15 more typically about 5% to about 25% by weight.

4. Other Additives

Other additives conventionally used in pharmaceutical compositions can be included, and these additives are well known in the art. Such additives include antioxidants, preservatives, chelating agents, viscomodulators, tonicifiers, flavorants, colorants odorants, 20 opacifiers, suspending agents, binders, and mixtures thereof. The amounts of such additives can be readily determined by one skilled in the art, according to the particular properties desired.

5. Dosage Forms

25 The pharmaceutical compositions of the present invention can be provided in the form of a solution concentrate; *i.e.*, a composition as described above, and intended to be dispersed with water, either prior to administration, in the form of a drink, or dispersed *in vivo*. Alternatively, the compositions can be provided in the form of a diluted concentrate (*i.e.*, an aqueous dispersion), a semi-solid dispersion or a solid dispersion. If desired, the compositions may be encapsulated in a hard or soft gelatin capsule, a starch capsule or an 30 enteric coated capsule. The term "enteric coated capsule" as used herein means a capsule coated with a coating resistant to acid; *i.e.*, an acid resistant enteric coating. Although solubilizers are typically used to enhance the solubility of a hydrophobic therapeutic agent, they may also render the compositions more suitable for encapsulation in hard or soft gelatin

1 capsules. Thus, the use of a solubilizer such as those described above is particularly preferred in capsule dosage forms of the pharmaceutical compositions. If present, these solubilizers should be added in amounts sufficient to impart to the compositions the desired solubility enhancement or encapsulation properties.

5 Although formulations specifically suited to oral administration are presently preferred, the compositions of the present invention can also be formulated for topical, transdermal, ocular, pulmonary, vaginal, rectal, transmucosal or parenteral administration, in the form of a triglyceride-free cream, lotion, ointment, suppository, gel or the like. If such a formulation is desired, other additives may be included, such as are well-known in the art, to
10 impart the desired consistency and other properties to the formulation. The compositions of the present invention can also be formulated as a spray or an aerosol. In particular, the compositions may be formulated as a sprayable solution, and such formulation is particularly useful for spraying to coat a multiparticulate carrier, such as a bead. Such multiparticulate carriers are well known in the art.

15 6. Preparation of Pharmaceutical Compositions

The pharmaceutical compositions of the present invention can be prepared by conventional methods well known to those skilled in the art. Of course, the specific method of preparation will depend upon the ultimate dosage form. For dosage forms substantially free of water, *i.e.*, when the composition is provided in a pre-concentrated form for later
20 dispersion in an aqueous system, the composition is prepared by simple mixing of the components to form a pre-concentrate. The mixing process can be aided by gentle heating, if desired. For compositions in the form of an aqueous dispersion, the pre-concentrate form is prepared, then the appropriate amount of purified water is added. Upon gentle mixing, a clear aqueous dispersion is formed. If any water-soluble additives are included, these may be
25 added first as part of the pre-concentrate, or added later to the clear aqueous dispersion, as desired.

In another embodiment, the present invention includes a multi-phase dispersion. In this embodiment, a pharmaceutical composition includes a carrier which forms a clear aqueous dispersion upon mixing with an aqueous solution, and an additional amount of non-
30 solubilized hydrophobic therapeutic agent. Thus, the term "multi-phase" as used herein to describe these compositions of the present invention means a composition which when mixed with an aqueous solution forms a clear aqueous phase and a particulate dispersion phase. The carrier is as described above, and can include any of the surfactants, hydrophobic therapeutic

1 agents, solubilizers and additives previously described. An additional amount of
hydrophobic therapeutic agent is included in the composition. This additional amount is not
solubilized by the carrier, and upon mixing with an aqueous system is present as a separate
dispersion phase. The additional amount is optionally a milled, micronized, or precipitated
5 form. Thus, upon dilution, the composition contains two phases: a clear aqueous dispersion
of the hydrophilic and hydrophobic surfactants containing a first, solubilized amount of the
hydrophobic therapeutic agent, and a second, non-solubilized amount of the hydrophobic
therapeutic agent dispersed therein. It should be emphasized that the resultant multi-phase
dispersion will not have the optical clarity of a dispersion in which the hydrophobic
10 therapeutic agent is fully solubilized, but will appear to be cloudy, due to the presence of the
non-solubilized phase. Such a formulation may be useful, for example, when the desired
dosage of a hydrophobic therapeutic agent exceeds that which can be solubilized in the
carrier of the present invention. The formulation may also contain additives, as described
above.

15 One skilled in the art will appreciate that a hydrophobic therapeutic agent may have a
greater solubility in the pre-concentrate carrier than in the aqueous dispersion, so that meta-
stable, supersaturated solutions having apparent optical clarity but containing a hydrophobic
therapeutic agent in an amount in excess of its solubility in the aqueous dispersion can be
formed. Such super-saturated solutions, whether characterized as clear aqueous dispersions
20 (as initially formed) or as multi-phase solutions (as would be expected if the meta-stable state
breaks down), are also within the scope of the present invention.

The multi-phase formulation can be prepared by the methods described above. A pre-
concentrate is prepared by simple mixing of the components, with the aid of gentle heating, if
desired. It is convenient to consider the hydrophobic therapeutic agent as divided into two
25 portions, a first solubilizable portion which will be solubilized by the carrier and contained
within the clear aqueous dispersion upon dilution, and a second non-solubilizable portion
which will remain non-solubilized. When the ultimate dosage form is non-aqueous, the first
and second portions of the hydrophobic therapeutic agent are both included in the pre-
concentrate mixture. When the ultimate dosage form is aqueous, the composition can be
30 prepared in the same manner, and upon dilution in an aqueous system, the composition will
form the two phases as described above, with the second non-solubilizable portion of the
hydrophobic therapeutic agent dispersed or suspended in the aqueous system, and the first
solubilizable portion of the hydrophobic therapeutic agent solubilized in the mixed surfactant

1 carrier. Alternatively, when the ultimate dosage form is aqueous, the pre-concentrate can be prepared including only the first, solubilizable portion of the hydrophobic therapeutic agent. This pre-concentrate can then be diluted in an aqueous system to form a clear aqueous dispersion, to which is then added the second, non-solubilizable portion of the hydrophobic
5 therapeutic agent to form a multi-phase aqueous composition.

The amount of hydrophobic therapeutic agent included in the pharmaceutical compositions of the present invention can be any amount desired by the formulator, up to the maximum amount that can be solubilized or suspended in a given carrier system. In general, the amount of hydrophobic therapeutic agent will be about 0.1% to about 60% by weight,
10 based on the total weight of the pharmaceutical composition. In another aspect of the invention, described below, excess hydrophobic therapeutic agent can also be added, in a multi-phase dispersion.

B. Methods of Improved Delivery

15 In another aspect, the present invention relates to methods of improving delivery of hydrophobic therapeutic agents in an animal by administering to the animal a dosage form of the pharmaceutical compositions described herein. Preferably the animal is a mammal, and more preferably, a human. It has been found that the pharmaceutical compositions of the present invention when administered to an animal enable the hydrophobic therapeutic agent contained therein to be absorbed more rapidly than in conventional pharmaceutical
20 compositions. Thus, in this aspect the invention relates to a method of increasing the rate of and/or extent of bioabsorption of a hydrophobic therapeutic agent by administering the hydrophobic therapeutic agent to an animal in the pharmaceutical compositions described herein.

C. Characteristics of the Pharmaceutical Compositions

25 The dispersions formed upon dilution of the pharmaceutical compositions of the present invention have the following characteristics:

Rapid formation: upon dilution with an aqueous solution, the carrier forms a clear dispersion very rapidly; *i.e.*, the clear dispersion appears to form instantaneously.

30 Optical clarity: the dispersions are essentially optically clear to the naked eye, and show no readily observable signs of heterogeneity, such as turbidity or cloudiness. More quantitatively, dispersions of the pharmaceutical compositions of the present invention show a mono-modal distribution of very small particles sizes, typically 20 nm or less in average diameter; absorbances of less than about 0.3, typically less than about 0.1, at 10X dilution;

1 and absorbances of less than about 0.1, typically less than about 0.01, at 100X dilution, as
described more fully in the Examples herein. In the multi-phase embodiment of the
compositions described herein, it should be appreciated that the optical clarity of the aqueous
carrier dispersion phase will be obscured by the dispersed particulate non-solubilized
5 hydrophobic therapeutic agent.

Robustness to dilution: the dispersions are surprisingly stable to dilution in aqueous
solution, including aqueous solutions simulating physiological fluids such as enzyme-free
simulated gastric fluid (SGF) and enzyme-free simulated intestinal fluid (SIF). The
hydrophobic therapeutic agent remains solubilized for at least the period of time relevant for
10 absorption.

Triglyceride-free: It is a particular feature of the present invention that the
pharmaceutical compositions are substantially triglyceride-free. The term "triglyceride" as
used herein means glycerol triesters of C₆ to about C₂₅ fatty acids. Unlike conventional
compositions such as oil-based solutions, emulsions, and microemulsions, which rely on the
15 solubilizing power of triglycerides, the present compositions surprisingly solubilize
hydrophobic therapeutic agents using combinations of substantially triglyceride-free
surfactants.

As used herein, the term "substantially triglyceride-free" means compositions which
contain triglycerides, if at all, only as minor components or impurities in surfactant mixtures.
20 It is well known in the art that commercially available surfactants often are complex mixtures
of compounds. For example, one preferred surfactant is Capmul® GMO-K, a widely-used
blend of glyceryl mono- and dioleates. Due to difficulties in separating complex product
mixtures, however, a typical lot of Capmul® GMO-K, as reported by the manufacturer's
certificate of analysis, contains the following distribution of glyceryl esters, in percent by
25 weight based on the total weight of glyceryl esters:

Palmitic acid	3.3%
Stearic acid	4.0%
Oleic acid	81.0%
Linoleic acid	9.7%
30 Linolenic acid	0.3%

In addition, the surfactant mixture in the particular lot reported contains 0.10% water and
0.95% free, unesterified glycerol. These specific percentages are expected to vary, lot-by-lot,
as well, and it is expected that commercial surfactant products will generally possess similar

1 variability, regardless of the specific major component and the specific manufacturer. Thus,
the present invention does not include surfactants which contain triglycerides as an intended
component. Indeed, such surfactants are not common, since triglycerides themselves have no
surfactant properties. However, it should be appreciated that the present invention does not
5 exclude the use of surfactant products which contain small amounts of triglycerides as
impurities or as unreacted starting material. It is expected that commercial mixtures suitable
for use in the present invention may contain as much as 5% triglycerides by weight as
unintended components. Thus, "substantially triglyceride-free" should be understood as
meaning free of added triglycerides, and containing less than 5%, preferably essentially 0%,
10 triglyceride impurities.

Without wishing to be bound by theory, it is believed that the observed properties of
the clear, aqueous dispersions formed by the compositions of the present invention are
consistent with, and best explained by, the formation of mixed micelles of the hydrophobic
and hydrophilic surfactants, with the hydrophobic therapeutic agent solubilized by the
15 micelles. It should be emphasized that these dispersions are characterized by the properties
described herein, regardless of the precise microscopic physical form of the dispersed
particles. Nevertheless, in order to more fully explain the invention, and to illustrate its
unexpected and important advantages, the following discussion is offered in terms consistent
with the theoretical principles believed to be correct.

20 It is believed that the hydrophobic and hydrophilic surfactants form mixed micelles in
aqueous solution. In this model, each micelle is composed of molecules (or ions) of both the
hydrophilic and hydrophobic surfactants. Depending upon the detailed three-dimensional
structure of the hydrophobic therapeutic agent, its distribution of polar moieties, if any, its
polarizability in local regions, and other molecule-specific and complex factors, the
25 hydrophobic therapeutic agent may be distributed in any part of the micelle, such as near the
outer, more hydrophilic region, near the inner, more hydrophobic region, or at various points
in between. Further, it is known that micelles exist in dynamic equilibrium with their
component molecules, and it is expected that this equilibrium will include dynamic
redistribution of the hydrophobic therapeutic agent.

30 As discussed above, triglyceride-containing formulations suffer the disadvantage that
bioabsorption of the hydrophobic therapeutic agents contained therein is dependent upon
enzymatic degradation (lipolysis) of the triglyceride components. The pharmaceutical
compositions of the present invention, however, are substantially free of triglycerides, and

1 thus do not depend upon lipolysis to enable release of the hydrophobic therapeutic agent for bioabsorption. The hydrophobic therapeutic agent is in a dynamic equilibrium between the free compound in solution and the solubilized compound, thus promoting rapid release.

5 The unique pharmaceutical compositions of the present invention present a number of significant and unexpected advantages, including:

Efficient transport: The particle sizes in the aqueous dispersions of the present invention are much smaller, typically less than 20 nm, than the larger particles characteristic of vesicular, emulsion or microemulsion phases, and the particle size distribution is mono-modal and narrow. This reduced and more uniform size enables more efficient drug transport
10 through the intestinal aqueous boundary layer, and through the absorptive brush border membrane. More efficient transport to absorptive sites leads to improved and more consistent absorption of hydrophobic therapeutic agents.

Non-dependence on lipolysis: The lack of triglyceride components provides pharmaceutical compositions not dependent upon lipolysis, and upon the many poorly
15 characterized factors which affect the rate and extent of lipolysis, for effective presentation of a hydrophobic therapeutic agent to an absorptive site. Such factors include the presence of composition components which may inhibit lipolysis; patient conditions which limit production of lipase, such as pancreatic lipase secretory diseases; and dependence of lipolysis on stomach pH, endogenous calcium concentration, and presence of co-lipase or other
20 digestion enzymes. The lack of lipolysis dependence further provides transport which does not suffer from any lag time between administration and absorption caused by the lipolysis process, enabling a more rapid onset of therapeutic action and better bioperformance characteristics. In addition, pharmaceutical compositions of the present invention can make use of hydrophilic surfactants which might otherwise be avoided or limited due to their
25 potential lipolysis inhibiting effects.

Non-dependence on bile and meal fat contents: Due to the higher solubilization potential over bile salt micelles, the present compositions are less dependent on endogenous bile and bile related patient disease states, and meal fat contents. These advantages overcome meal-dependent absorption problems caused by poor patient compliance with meal-dosage
30 restrictions.

Superior solubilization: The surfactant combinations used in compositions of the present invention enable superior loading capacity over conventional micelle formulations. In addition, the particular combination of surfactants used can be optimized for a specific

1 hydrophobic therapeutic agent to more closely match the polarity distribution of the
therapeutic agent, resulting in still further enhanced solubilization.

5 Faster dissolution and release: Due to the robustness of compositions of the present
invention to dilution, the hydrophobic therapeutic agents remain solubilized and thus do not
suffer problems of precipitation of the therapeutic agent in the time frame relevant for
absorption. In addition, the therapeutic agent is presented in small particle carriers, and is not
limited in dilution rate by entrapment in emulsion carriers. These factors avoid liabilities
associated with the poor partitioning of lipid solubilized drug in to the aqueous phase, such as
large emulsion droplet surface area, and high interfacial transfer resistance, and enable rapid
10 completion of the critical partitioning step.

Consistent performance: Aqueous dispersions of the present invention are
thermodynamically stable for the time period relevant for absorption, and can be more
predictably reproduced, thereby limiting variability in bioavailability-- a particularly
important advantage for therapeutic agents with a narrow therapeutic index.

15 Efficient release: The compositions of the present invention are designed with
components that help to keep the hydrophobic therapeutic agent solubilized for transport to
the absorption site, but readily available for absorption, thus providing a more efficient
transport and release.

20 Less prone to gastric emptying delays: Unlike triglyceride-containing formulations,
the present compositions are less prone to gastric emptying delays, resulting in faster
absorption. Further, the particles in dispersions of the present invention are less prone to
unwanted retention in the gastro-intestinal tract.

25 Small size: Because of the small particle size in aqueous dispersion, the
pharmaceutical compositions of the present invention allow for faster transport of the
hydrophobic therapeutic agent through the aqueous boundary layer.

These and other advantages of the present invention, as well as aspects of preferred
embodiments, are illustrated more fully in the Examples which follow.

EXAMPLES

Example 1: Preparation of Compositions

A simple pre-concentrate of a hydrophobic surfactant and a hydrophilic surfactant is prepared as follows. Predetermined weighed amounts of hydrophilic and hydrophobic surfactants are stirred together to form a homogeneous mixture. For surfactant combinations that are poorly miscible, the mixture can be gently heated to aid in formation of the homogeneous mixture. A chosen hydrophobic therapeutic agent in a predetermined amount is added and stirred until solubilized. Optionally, solubilizers or additives are included by simple mixing.

To form an aqueous dispersion of the pre-concentrate, a predetermined amount of purified water, buffer solution, or aqueous simulated physiological solution, is added to the pre-concentrate, and the resultant mixture is stirred to form a clear, aqueous dispersion.

Example 2: Surfactant Combinations Giving Clear Aqueous Dispersions

Surfactant mixtures giving clear, aqueous dispersions were prepared according to the method of Example 1. Seven hydrophilic surfactants and sixteen hydrophobic surfactants were used to produce approximately one hundred clear aqueous dispersions suitable for use in the present invention. For simplicity, no hydrophobic therapeutic agent was included in these compositions, since it is believed that the presence of the hydrophobic therapeutic agent does not substantially affect the clear, aqueous nature of composition. For the same reason, these compositions were free of additional solubilizers and other additives.

Multiple solutions were prepared for each surfactant combination, to determine the approximate maximum amount of hydrophobic therapeutic agent giving a clear aqueous dispersion with a given amount of hydrophilic therapeutic agent. Thus, for each gram of the hydrophilic surfactant, a predetermined amount of hydrophobic agent was used to prepare a 10X aqueous dispersion. If the dispersion appeared to be optically clear, a new dispersion was prepared according to Example 1, using a larger amount of hydrophobic surfactant. Similarly, if the dispersion appeared to be cloudy, a new dispersion was prepared using a smaller amount of hydrophobic surfactant. The results are shown in Table 19.

TABLE 19: Surfactant Combinations Giving Clear Dispersions

	Hydrophilic Surfactant	PEG-35 Castor Oil (Incrocas 35)	PEG-40H Castor Oil (Cremophor RH-40)	Polysorbate-20 (Tween 20)	Polysorbate 80 (Tween 80)	PEG-60 Corn Oil (Crovol M-70)	PEG-8 Capric /Caprylic (Labrasol)	PEG-25 Glyceryl trioleate (Tagat TO)
5	Hydrophobic Surfactant							
10	Glyceryl/ Propylene Glycol Oleate (Arlacel 186)	20	20	20	8	15	25	10
	Glyceryl Oleate (Peceol)	15	40	10	12	10	35	10
15	Acetylated Monoglycerides (Myvacet 9-45)	80	80	20	15	10	10	10
	PEG-6 Corn Oil (Labrafil M2125CS)	50	95	10	10	20	10	10
20	Sorbitan Monooleate (Span 80)	25	65	5	5	20	15	10
	Sorbitan Monolaurate (Arlacel 20)	30	20	20	10	15	30	10
25	Polyglyceryl oleate (Plurol Oleique CC497)	10	5	35	10	10	35	10
	Propylene Glycol Laurate (Lauroglycol FCC)	10	55	35	20	15	35	10
30	Glyceryl Caprylate / Caprate (Capmul MCM)	10	50	20	25	25	20	10

1	PEG-20 Corn Oil (Crovol M-40)	35	40	40	25	30	90	10
5	PEG-20 Almond Oil (Crovol A-40)	30	35	40	25	30	90	10
	Mono/diglycerid es of Caprylic Acid (Imwitor 988)	50	50	60	25	25	30	10
10	PEG-4-lauryl ether (Brij 30)	40	45	95	70	*	90	10
	PEG-3-oleyl ether (Volpo 3)	20	30	25	20	20	25	10
15	Glyceryl mono/dioleate (Capmul GMO- K)	*	10	*	*	10	25	10
	Ethyl Oleate (Crodamol EO)	40	60	10	10	60	10	10

20 * This combination was not tested.

Each entry in the Table represents the approximate maximum number of grams of hydrophobic surfactant per 100 g of hydrophilic surfactant giving acceptable optical clarity. The numbers in the Table are illustrative only, and it is expected that further optimization of the surfactant systems with solubilizers, co-surfactants, and other additives will give still
25 higher numbers.

Example 3: Compositions Containing Solubilizers

The procedure of Example 2 was repeated for compositions containing PEG-40 hydrogenated castor oil (Cremophor RH 40) as the hydrophilic surfactant, with eight different hydrophobic surfactants, and four different solubilizers, to study the effect of solubilizer on
30 the relative amounts of hydrophobic and hydrophilic surfactants giving clear aqueous dispersions. In each case, the amount of solubilizer was held constant at 20% by weight, based on the total weight of the two surfactants. The results are shown in Table 20. As in Example 2, the numbers in the Table represent the approximate maximum number of grams

1 of hydrophobic surfactant per 100 g of hydrophilic surfactant giving a clear aqueous dispersion. For convenience, the corresponding entries from Table 19 (with no solubilizer present) are reproduced in Table 20 in the column labeled "none."

Table 20: Effect of Solubilizer on Hydrophobic Surfactant Amounts

Hydrophobic Surfactant	Hydrophilic Surfactant (Cremophor RH40) + 20% Solubilizer				
	(None)	Triacetin	Ethanol	PEG-400	Glycofurol
10 Glyceryl/ Propylene Glycol Oleate (Arlacel 186)	20	28	25	25	25
Glyceryl Oleate (Peceol)	40	40	42	40	44
Sorbitan Monooleate (Span 80)	65	40	40	25	30
15 Sorbitan Monolaurate (Span 20)	20	65	*	*	65
PEG-6 Corn Oil (Labrafil M2125CS)	95	95	*	95	*
20 Acetylated Monoglyceride (Myvacet 9-45)	80	80	80	80	80
Ethyl Oleate (Crodamol EO)	60	60	60	*	60
25 Mono/diglycerides of Caprylic Acid (Imwitor 988)	50	80	*	*	75

* This combination was not tested.

As is clear from the data in the Table, the effect of added solubilizer on the relative amount of hydrophobic surfactant that can be used varies considerably. For some surfactant combinations, the added solubilizer has a dramatic effect on the amount of hydrophobic surfactant (e.g., Span 20, Imwitor 988). In other systems, the effect is moderate (Arlacel 186, Peceol) or negligible (Crodamol EO, Myvacet 9-45). In the one case of Span 80, the presence of the solubilizer actually decreases the amount of hydrophobic surfactant that can be used.

Example 4: Compositions Containing Solubilizers

Example 3 was repeated, this time choosing a single hydrophobic surfactant (Arlacel 186) and three different hydrophilic surfactants, with addition of either ethanol or triacetin (20% by weight, based on the total weight of the two surfactants). The results are shown in Table 21. The corresponding entry from Table 19 (with no solubilizer present) is included in Table 21 for reference.

Table 21: Effect of Solubilizer on Hydrophobic Surfactant Amounts

Hydrophilic Surfactant	Hydrophobic Surfactant (Arlacel 186) + 20% Solubilizer		
	(None)	Ethanol	Triacetin
PEG-60 Corn Oil (Crovol M-70)	15	20	20
PEG-35 Castor Oil (Incrocas 35)	20	25	25
Polysorbate 20 (Tween 20)	20	25	25

In each case, a moderate increase (20%) in the relative amount of hydrophobic surfactant was observed.

Example 5: Effect of Solubilizer Concentration

The procedure of Example 3 was repeated, with the following differences. A single hydrophilic surfactant (Cremophor RH-40) and hydrophobic surfactant (Arlacel 186) were chosen, to examine the effect of increased solubilizer concentration. For each of the four solubilizers tested at 20% concentrations in Example 3 (Table 20) plus an additional solubilizer (propylene glycol), compositions were tested at a solubilizer concentration of 50% by weight, based on the total weight of the surfactant pair. As in each of the previous examples, the numbers in Table 22 represent the maximum hydrophobic surfactant concentration giving a clear aqueous dispersion. Note that the "0" column in Table 22 reproduces the numbers shown in Table 19 (no solubilizer), and the "20%" column reproduces the numbers in Table 20, with the value for propylene glycol also supplied.

Table 22: Effect of Solubilizer Concentration on Hydrophobic Surfactant Amounts*

Solubilizer	Weight Percent of Solubilizer		
	0	20	50
PEG-400	20	25	25
Propylene Glycol	20	28	30
Triacetin	20	28	25
Ethanol	20	25	30
Glycofurol	20	25	30

* for an Arlacel 186 (hydrophobic) - Cremophor RH-40 (hydrophilic) surfactant pair

As the Table shows, increasing the amount of solubilizer has a small to moderate effect on the amount of hydrophobic surfactant that can be present in a clear aqueous dispersion. It should be appreciated that the data equivalently show that very large amounts of solubilizer can be used, without detrimental effect on the ability of the surfactant system to form a clear, aqueous dispersion.

Example 6: Effect of High Solubilizer Concentration and Solubilizer Mixtures

Example 5 was repeated, using the same surfactant pair, but with an 80% concentration of solubilizer, based on the total weight of the surfactants. The 80% solubilizer was either PEG-400, or a mixture of PEG-400 and one of three alcohols or polyols. The results are shown in Table 23, with the numbers in the Table having the same meaning as in the previous Examples.

Table 23: Large Solubilizer Concentrations and Solubilizer Mixtures*

(no solubilizer)	80% PEG-400	60% PEG-400 + 20% Glycerol	60% PEG-400 + 20% Propylene Glycol	60% PEG-400 + 20% Isopropanol
20	25	25	25	25

* for an Arlacel 186 (hydrophobic) - Cremophor RH-40 (hydrophilic) surfactant pair

It is clear from the data in the Table that very high concentrations of solubilizers, as well as mixtures of solubilizers, can be used effectively in the clear aqueous dispersions of the present invention.

Examples 7-12: Average Particle Size

In order to more quantitatively characterize the clear aqueous dispersions of the present invention, particle sizes were measured for several compositions of the present invention. For simplicity, the measurement were made for the dispersed carrier, in the absence of a hydrophobic therapeutic agent. In this Example, formulations were prepared as in Example 1, and diluted to form 10X or 100X aqueous dispersions. Each of the resulting dispersions was observed to be optically clear to the naked eye. Average particle sizes were measured with a Nicomp Particle Size Analyzer (Particle Size Systems, Inc., Santa Barbara, CA). The results of these measurements are shown in Table 24.

Table 24: Average Particle Size

Examp e No.	Formula		Surfacta nt Ratio*	Dilution	Observatio n	Particle Size (nm) ± S.D.**
7	Tween 80 Lauroglycol FCC	520 mg 50 mg	9.6	100X	very clear solution	6.5 ± 1.1
8	Tween 80 Capmul MCM	500 mg 73 mg	15	10X	very clear solution	8.1 ± 1.6
9	Cremophor RH- 40 Peceol	530 mg 150 mg	28	100X	clear solution	12.4 ± 3.0
10	Cremophor RH- 40 Plurol Oleique CC497	500 mg 10 mg	2.0	100X	clear solution	14.7 ± 3.0
11	Cremophor RH- 40 Lauroglycol FCC	550 mg 200 mg	36	100X	clear solution	14.3 ± 2.5
12	Cremophor RH- 40 Capmul MCM	500 mg 200 mg	40	100X	clear solution	12.6 ± 2.9

* grams of hydrophobic surfactant per 100 g of hydrophilic surfactant

**** standard deviation**

As the data show, the compositions of the present invention produce clear, aqueous dispersions, with no visible cloudiness. The particle size distribution shows very small

particles, with average diameters of from about 6 to about 15 nm. The distribution is mono-modal, with a standard deviation of approximately 20%, indicating a highly uniform distribution of very small particles. This particle size distribution is consistent with a solution of particles of micellar structure, although the invention is not limited by any particular theoretical framework.

Comparative Examples C1-C5: Optical Clarity and Particle Sizes of Compositions
Not Forming Clear Aqueous Dispersions

For comparison to the clear aqueous dispersions of the present invention, several compositions were prepared having hydrophobic surfactant concentrations higher than those suitable for forming clear aqueous dispersions. These compositions were prepared by weighing the components and mixing well, with gentle warming. The compositions were then diluted 10X to form dispersions, and these dispersions were subjected to the particle size measurements as described in Example 7. The results are shown in Table 25. For direct comparison with the compositions of the present invention, Examples 7, 9, 10, 11 and 12 are shown next to the corresponding comparative compositions.

Table 25: Optical Clarity and Particle Size

Example No.	Surfactants	Surfactant Ratio*	Observation	Particle Size (nm)**	
				Mean 1	Mean 2
C1	Tween 80 Lauroglycol FCC	67	milky solution	26.6	209
7	Tween 80 Lauroglycol FCC	9.6	very clear solution	6.5	---
C2	Cremophor RH-40 Peceol	67	milky solution	25	116
9	Cremophor RH-40 Peceol	28	clear solution	8.1	---
C3	Cremophor RH-40 Plurol Oleique CC497	67	milky solution	16.5	102
10	Cremophor RH-40 Plurol Oleique CC497	2.0	clear solution	12.4	---

1	C4	Cremophor RH-40 Lauroglycol FCC	69	hazy solution	17.1	45.3
	11	Cremophor RH-40 Lauroglycol FCC	36	clear solution	14.3	
5	C5	Cremophor RH-40 Capmul MCM	67	milky solution	11.6	176
	12	Cremophor RH-40 Capmul MCM	40	clear solution	12.6	---

* grams of hydrophobic surfactant per 100 g of hydrophilic surfactant

** two means are reported for bimodal distributions

In addition to the compositions shown in the Table, compositions containing Tween 80 and Plurol Oleique CC497, Tween 80 and Peceol, and Tween 80 and Capmul MCM were prepared at a surfactant ratio of 67 g hydrophobic surfactant per 100 g hydrophilic surfactant.

Particle sizes were not measured for these compositions, but each was observed to form a milky or hazy aqueous dispersion.

As the data show, compositions having excessive amounts of hydrophobic surfactant form milky or hazy solutions, whereas those of the present invention form clear solutions. In addition, the particle size distributions of the milky solutions are bimodal, in contrast to the mono-modal solutions of the corresponding clear solutions. These bimodal particle size distributions show a first mode having a small mean particle size of about 12 to about 27 nm, and a second mode having particle sizes of up to more than 200 nm. Thus, compositions having excessive hydrophobic surfactant are heterogeneous (multi-phasic), non-clear dispersions, having a complex bimodal distribution of particles of two distinct size ranges. In contrast, compositions of the present invention are homogeneous (single phase), clear dispersion, having a mono-modal distribution of very small particle sizes.

Examples 13-42: Spectroscopic Characterization of Optical Clarity

The optical clarity of aqueous dispersions of the present invention was measured spectroscopically. Compositions were prepared according to Example 1, and diluted to 10X and 100X solutions. The specific compositions measured also include a solubilizer, to further illustrate preferred aspects of the invention. In addition, several of the compositions illustrate compositions according to the present invention wherein either the hydrophilic surfactant

(Examples 20 and 27) or the hydrophobic surfactant (Examples 41 and 42) itself is a mixture of surfactants.

The absorbance of each solution was measured at 400.2 nm, using a purified water standard, and the results are shown in Table 26.

Table 26: Spectroscopic Characterization of Optical Clarity

Example No.	Formulation		Absorbance (400.2 nm)	
			10X	100X
13	Cremophor RH-40 Myvacet 9-45 Ethyl Alcohol	430 mg 310 mg 210 mg	0.407	0.099
14	Cremophor RH-40 Peceol Ethyl Alcohol	610 mg 160 mg 200 mg	0.299	0.055
15	15 Cremophor RH-40 Span 80 Triacetin	540 mg 260 mg 220 mg	0.655	0.076
16	16 Incrocas 35 Myvacet 9-45 Ethyl Alcohol	470 mg 250 mg 220 mg	0.158	0.038
17	17 Incrocas 35 Imwitor 988 Triacetin	510 mg 220 mg 200 mg	0.064	0.009
18	18 Tween 20 Lauroglycol FCC Glycofurol	570 mg 140 mg 220 mg	0.031	0.003
19	19 Crovol M70 Crovol M40 Ethyl Alcohol	610 mg 120 mg 200 mg	0.049	0.006
20	20 Cremophor RH-40 Labrasol Capmul GMO-K Triacetin	250 mg 250 mg 110 mg 100 mg	0.028	0.008
21	21 Cremophor RH-40 Lauroglycol FCC Ethyl Alcohol	220 mg 200 mg 75 mg	0.114	0.018

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22	Tween 80 Capmul MCM Ethyl Alcohol	170 mg 30 mg 38 mg	0.050	0.008
23	Cremophor RH-40 Capmul MCM Ethyl Alcohol	550 mg 80 mg 53 mg	0.029	0.006
24	Cremophor RH-40 Peceol Ethyl Alcohol	230 mg 70 mg 54 mg	0.187	0.020
25	Cremophor RH-40 Plurol Oleique CC497 Ethyl Alcohol	500 mg 10 mg 11 mg	0.028	0.005
26	Tween 80 Lauroglycol FCC Ethyl Alcohol	180 mg 20 mg 37 mg	0.036	0.003
27	Tween 80 Labrasol Arlacel 186 Ethyl Alcohol	420 mg 330 mg 54 mg 140 mg	0.036	0.009
28	Tagat O2 PGMG-03 Ethyl Alcohol	500 mg 50 mg 100 mg	0.077	0.005
29	Incrocas 35 Gelucire 44/14 Triacetin	250 mg 150 mg 94 mg	0.053	0.005
30	Cremophor RH-40 Labrafil Ethyl Alcohol	270 mg 170 mg 100 mg	0.232	0.047
31	Crovol M-70 Labrafil Triacetin	380 mg 50 mg 100 mg	0.064	0.011
32	Cremophor RH-40 Peceol Triacetin	300 mg 110 mg 110 mg	0.163	0.034
33	Tween 20 Lauroglycol FCC	340 mg 110 mg	0.038	0.005

1		Glycofurol	100 mg		
	34	Incrocas-35 Labrafil Ethyl Alcohol	310 mg 110 mg 100 mg	0.101	0.020
5	35	Cremophor RH-40 Span 80 Triacetin	300 mg 130 mg 100 mg	0.908	0.114
	36	Cremophor RH-40 Arlacel 186 Propylene Glycol	510 mg 58 mg 55 mg	0.039	0.008
10	37	Cremophor RH-40 Peceol Propylene Glycol	510 mg 140 mg 58 mg	0.440	0.100
	38	Cremophor RH-40 Labrafil M2125CS Propylene Glycol	500 mg 400 mg 88 mg	0.411	0.107
15	39	Cremophor RH-40 Span 80 Propylene Glycol	550 mg 220 mg 78 mg	0.715	0.106
	40	Cremophor RH-40 Crodamol Propylene Glycol	500 mg 280 mg 100 mg	0.547	0.147
20	41	Cremophor RH-40 Labrafil M2125CS Span 80 Ethyl Alcohol	550 mg 340 mg 200 mg 110 mg	0.419	0.055
25	42	Cremophor RH-40 Labrafil M2125CS Crovol M-40 Ethyl Alcohol	500 mg 270 mg 280 mg 100 mg	0.293	0.260

Ideally, a clear aqueous dispersion should have a very high transmittance, indicating little scattering of light by large particles. Absorbance and transmittance are related by the simple expression

$$A = -\log T$$

where A is absorbance, and T is the transmittance expressed as a decimal. Thus, preferred solutions of the present invention will have small absorbances. As noted above, in the

1 absence of true absorption (due to chromophores in solution), suitable clear aqueous
dispersions of the present invention should have an absorbance at 10X dilution of less than
about 0.3.

5 The data in Table 26 show 30 solutions, 22 of which have absorbances less than about
0.3 at 10X dilution. Of these solutions, 3 have absorbances between 0.2 and 0.3, 5 have
absorbances between 0.1 and 0.2, and 14 have absorbances less than 0.1. Thus, for the
majority of the solutions, absorbance provides an adequate measure of optical clarity.

Solutions having absorbances greater than 0.3 may still be suitable for use in the
present invention, as these are observed to have acceptable optical clarity by visual
10 examination. For these relatively high absorbance solutions, this simple spectroscopic
measure of optical clarity is inadequate, and other methods are more well-suited to assessing
optical clarity, such as visual observation and particle size. As an example, Example 37,
which shows an absorbance of 0.440, has a surfactant ratio of 27, well below the value of 40
shown in Table 19, and is observed to be a clear solution. This same composition, without
15 the additional solubilizer, is shown in Example 9 at a surfactant ratio of 28 to have a mono-
modal, narrow particle size distribution, at an average particle size of 12.4 nm. It should be
appreciated that direct particle size measurement and absorbance measurement are different
ways of assessing optical clarity, and provide alternative criteria for quantifying clarity.
However, it is believed that the simple, qualitative visual observation of optical clarity is a
20 sufficient measure of suitable clarity for use in the present invention, particularly so since
compositions outside the scope of the invention show marked and unmistakable cloudiness
without recourse to quantitative measurement (*See, e.g., Comparative Example 1*).

Comparative Examples C6-C12: Spectroscopic Characterization of Compositions
Not Forming Clear Aqueous Dispersions

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For comparison to the clear aqueous dispersions of the present invention,
compositions observed to be milky or cloudy were characterized by absorption, as in
Examples 13-42. Where available, results for comparable solutions from Examples 13-42 are
reproduced for comparison. In such cases, where a given surfactant combination is presented
30 in Examples 13-42 more than once (with different solubilizer concentrations), the
composition having the lowest solubilizer concentration is chosen, to facilitate more direct
comparison. The results are shown in Table 27.

Table 27: Comparative Spectroscopic Characterization

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Example No.	Formulation		Absorbance (400.2 nm)	
			10X	100X
C6	Tween 80 Lauroglycol FCC	100 mg 67 mg	2.938	2.827
26	Tween 80 Lauroglycol FCC Ethyl Alcohol	180 mg 20 mg 37 mg	0.036	0.003
C7	Tween 80 Capmul MCM	100 mg 67 mg	0.980	0.932
22	Tween 80 Capmul MCM Ethyl Alcohol	170 mg 30 mg 38 mg	0.050	0.008
C8	Cremophor RH-40 Plurol Oleique CC497	100 mg 67 mg	2.886	1.595
25	Cremophor RH-40 Plurol Oleique CC497 Ethyl Alcohol	500 mg 10 mg 11 mg	0.028	0.005
C9	Cremophor RH-40 Peceol	100 mg 67 mg	2.892	1.507
24	Cremophor RH-40 Peceol Ethyl Alcohol	230 mg 70 mg 54 mg	0.187	0.020
C10	Cremophor RH-40 Capmul MCM	100 mg 67 mg	1.721	0.491
23	Cremophor RH-40 Capmul MCM Ethyl Alcohol	550 mg 80 mg 53 mg	0.029	0.006
C11	Tween 80 Plurol Oleique CC497	100 mg 67 mg	1.585	1.357
C12	Tween 80 Peceol	100 mg 67 mg	2.849	2.721

The data in the Table demonstrate that the clear aqueous dispersions of the present invention show very different absorptive behavior from compositions having excessive hydrophobic surfactant concentrations, having apparent absorbances (through scattering losses) lower by at least a factor of ten, and in some cases by a factor of more than one hundred.

Examples 43 and 44: Solubility of a Polyfunctional Hydrophobic Therapeutic Agent

The enhanced solubility of a typical polyfunctional hydrophobic therapeutic agent, cyclosporin, in the pharmaceutical compositions of the present invention was measured using a conventional "shake flask" method. Compositions were prepared and diluted to 10X and 100X as in Example 1, without including the therapeutic agent. The solutions were then provided with an excess of cyclosporin, and agitated to allow the cyclosporin to achieve an equilibrium partitioning between the solubilized phase and the non-solubilized dispersion phase. Concentration of the solubilized cyclosporin was then determined using standard HPLC techniques, optimized for the quantitative detection of cyclosporin. The results are shown in Table 28.

Table 28: Solubility of Cyclosporin in Clear Aqueous Dispersions

Example No.	Carrier Composition		Solubility ($\mu\text{g/mL}$)	
			10X Dilution	100X Dilution
43	Cremophor RH-40 Myvacet 9-45 Ethyl Alcohol	430 mg 321 mg 210 mg	13,205	1,008
44	Cremophor RH-40 Span 80 Triacetin	540 mg 260 mg 220 mg	11,945	1,127

This Example demonstrates the dramatically enhanced solubility of a hydrophobic therapeutic agent in the pharmaceutical compositions of the present invention.

Comparative Examples C13-C16: Solubility of a Polyfunctional Hydrophobic Therapeutic Agent

For comparison, the solubility experiment of Examples 43-44 was performed on four standard aqueous solutions. The first comparison solution was purified water with no additives. Next, a standard simulated intestinal fluid (SIF) was used, to simulate the in vivo

1 conditions to be encountered by the hydrophobic therapeutic agent. A third solution was
prepared with simulated intestinal fluid, plus an additional aliquot of 20 mM sodium
taurocholate (a bile salt); this solution is designated SIFB in Table 29. Finally, a fourth
5 solution was prepared with simulated intestinal fluid, 20 mM sodium taurocholate, and 5 mM
lecithin; this solution is designated SIFBL. The 20 mM bile salt and 5 mM lecithin
concentrations are believed to be representative of the average concentration of these
compounds encountered in the gastrointestinal tract. As in the previous Examples, these
comparison solutions were equilibrated with cyclosporin using the shake flask method, and
10 analyzed by HPLC. The results of these measurements are presented in Table 29.

Table 29: Solubility of Cyclosporin in Aqueous Solutions

Example No.	Solution	Solubility ($\mu\text{g/mL}$)
C13	Water	6
C14	SIF	6
C15	SIFB	49
C16	SIFBL	414
43-44 (average at 10X)	present invention	12,575

20 As the Table indicates, the solubility of the polyfunctional hydrophobic therapeutic
agent in the compositions of the present invention is far greater than its solubility in aqueous
and gastrointestinal aqueous solutions.

Examples 45-49: Solubility of a Lipophilic Hydrophobic Therapeutic Agent

25 The enhanced solubility of a typical lipophilic hydrophobic therapeutic agent,
progesterone, in the pharmaceutical compositions of the present invention was measured as
described in Examples 43-44. The results are shown in Table 30.

Table 30: Solubility of Progesterone in Clear Aqueous Dispersions

Example No.	Carrier Composition	Solubility ($\mu\text{g/mL}$)	
		10X Dilution	100X Dilution
45	Cremophor RH-40 1000 mg Arlacel 186 120 mg Propylene Glycol 110 mg	1100	200
46	Cremophor RH-40 1000 mg Peceol 240 mg Propylene Glycol 120 mg	1240	140
47	Cremophor RH-40 1000 mg Labrafil M2125CS 800 mg Propylene Glycol 180 mg	1760	190
48	Cremophor RH-40 1000 mg Span 80 350 mg Propylene Glycol 140 mg	1360	160
49	Cremophor RH-40 1000 mg Crodamol EO 600 mg Propylene Glycol 160 mg	1720	190

This Example demonstrates the dramatically enhanced solubility of a hydrophobic therapeutic agent in the pharmaceutical compositions of the present invention.

Comparative Examples C17-C20: Solubility of a Lipophilic Hydrophobic
Therapeutic Agent

For comparison, the solubility experiment of Comparative Examples C13-C16 was repeated, using progesterone instead of cyclosporin. The results of these measurements are presented in Table 31.

Table 31: Solubility of Progesterone in Aqueous Solutions

Example No.	Solution	Solubility ($\mu\text{g/mL}$)
C17	Water	6
C18	SIF	7-10
C19	SIFB	32-40

C20	SIFBL	80
45-49 (average at 10X)	Present invention	1436

As the Table indicates, the solubility of the lipophilic hydrophobic therapeutic agent in the compositions of the present invention is far greater than its solubility in aqueous and gastrointestinal aqueous solutions.

Examples 50-57: Aqueous Dilution Stability
of Compositions Containing a Polyfunctional Hydrophobic Therapeutic Agent

Compositions according to the present invention were prepared, with a typical polyfunctional hydrophobic therapeutic agent, cyclosporin, as the therapeutic agent. The compositions were prepared as described in Example 1, except that the ingredients were added in the order listed in Table 32. The pre-concentrates were diluted 100X with purified water, and a visual observation was made immediately after dilution. The solutions were then allowed to stand 6 hours to assess dilution stability, then the cyclosporin concentration in solution was measured, using a drug-specific HPLC assay. The results are shown in Table 32.

Table 32: Dilution Stability of Polyfunctional Therapeutic Agents

Example No.	Composition		Observation	Cyclosporin Concentration*
50	Cremophor RH-40 Myvacet 9-45 Ethyl Alcohol Cyclosporin	430 mg 310 mg 210 mg 99 mg	clear solution	121
51	Cremophor RH-40 Peceol Ethyl Alcohol Cyclosporin	610 mg 160 mg 200 mg 100 mg	clear solution	99
52	Cremophor RH-40 Span 80 Triacetin Cyclosporin	540 mg 260 mg 220 mg 97 mg	clear solution	114
53	Incrocas 35 Myvacet 9-45 Ethyl Alcohol	470 mg 250 mg 220 mg	clear solution	96

1		Cyclosporin	100 mg		
5	54	Cremophor RH-40 Arlacel 186 Propylene Glycol Ethanol Cyclosporin	660 mg 120 mg 100 mg 100 mg 100 mg	clear solution	105
	55	Cremophor RH-40 Arlacel 186 Propylene Glycol Cyclosporin	550 mg 120 mg 450 mg 100 mg	clear solution	102
10	56	Cremophor RH-40 Arlacel 186 Propylene Glycol Ethanol Cyclosporin	580 mg 120 mg 100 mg 100 mg 100 mg	clear solution	108
15	57	Gelucire 44/14 Incrocas 35 Glycofurol Cyclosporin	120 mg 200 mg 100 mg 100 mg	clear solution (at 37 °C)	108

* as a percentage of the initial cyclosporin concentration

The data in the Table indicate that large amounts of a polyfunctional hydrophobic therapeutic agent can be solubilized in the compositions of the present invention to produce clear, aqueous dispersions. These dispersions show no instability effects, such as hydrophobic therapeutic agent precipitation or particle agglomeration, upon standing.

Examples 58-74: Aqueous Dilution Stability of Compositions Containing a Lipophilic Hydrophobic Therapeutic Agent

Compositions according to the present invention were prepared, with a typical lipophilic hydrophobic therapeutic agent, progesterone, as the therapeutic agent. The compositions were prepared and analyzed as in Examples 50-57, and the results are shown in Table 33.

Table 33: Dilution Stability of Lipophilic Therapeutic Agents

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Example No.	Composition	Observation	Progesterone Concentration*
58	Cremophor RH-40 Arlacel 186 Propylene Glycol Progesterone	1000 mg 120 mg 110 mg 48 mg	very clear solution 99.1
59	Cremophor RH-40 Peceol Propylene Glycol Progesterone	1000 mg 240 mg 120 mg 48 mg	very clear solution 99.3
60	Cremophor RH-40 Labrafil Propylene Glycol Progesterone	1000 mg 800 mg 180 mg 45 mg	very clear solution 100.2
61	Cremophor RH-40 Span 80 Propylene Glycol Progesterone	1000 mg 350 mg 140 mg 50 mg	very clear solution 97.2
62	Cremophor RH-40 Crodamol EO Propylene Glycol Progesterone	1000 mg 600 mg 160 mg 48 mg	very clear solution 98.4
63	Cremophor RH-40 Labrafil M2125CS Ethyl Alcohol Progesterone	540 mg 350 mg 200 mg 42 mg	clear solution 104.4
64	Cremophor RH-40 Ethyl Oleate Ethyl Alcohol Progesterone	570 mg 260 mg 200 mg 42 mg	very slight tang blue color solution 106.1
65	Cremophor RH-40 Peceol Triacetin Progesterone	600 mg 210 mg 210 mg 42 mg	very slight tang blue color solution 104.6
66	Cremophor RH-40 Capmul MCM Triacetin Progesterone	600 mg 200 mg 200 mg 44 mg	very clear solution 97.7

1	67	Cremophor RH-40 Span 80 Triacetin Progesterone	590 mg 270 mg 210 mg 41 mg	clear solution	102.3
5	68	Crovol M-70 Labrafil M2125CS Triacetin Progesterone	760 mg 100 mg 200 mg 43 mg	very clear solution	104.6
10	69	Tween 20 Imwitor 988 Triacetin Progesterone	610 mg 300 mg 200 mg 45 mg	very slight tang blue color solution	98.0
	70	Tween 20 Lauroglycol FCC Glycofurol Progesterone	670 mg 170 mg 200 mg 43 mg	very clear solution	96.3
15	71	Incrocas 35 Labrafil M2125CS Ethyl Alcohol Progesterone	620 mg 220 mg 200 mg 43 mg	very clear solution	99.5
20	72	Incrocas 35 Span 20 Ethyl Alcohol Progesterone	660 mg 160 mg 210 mg 41 mg	very clear solution	105.9
	73	Cremophor RH-40 Arlacel 186 Propylene Glycol Progesterone	980 mg 130 mg 110 mg 110 mg	very clear supernatant	103.7
25	74	Cremophor RH-40 Labrafil Propylene Glycol Progesterone	520 mg 400 mg 110 mg 100 mg	very clear supernatant	103.1

* as a percentage of the initial progesterone concentration

The data in the Table indicate that a lipophilic hydrophobic therapeutic agent can be
 30 solubilized in the compositions of the present invention to produce clear, aqueous
 dispersions. These dispersions show no instability effects, such as hydrophobic therapeutic
 agent precipitation or particle agglomeration, upon standing.

1 Example 75: Enhancement of Bioabsorption

 Studies were performed to establish that the clear aqueous dispersions of the present invention facilitate an increased rate of bioabsorption of the hydrophobic therapeutic agent contained therein. The studies used a rat model with perfused intestinal loop along with
5 cannulation of the mesenteric vein. This unique methodology enabled assessment of the “true” absorption potential free of any systemic metabolic interference.

 A representative preconcentrate of the present invention containing a cyclosporin hydrophobic therapeutic agent was used. The composition had the following formulation:

10 Cyclosporine	0.140 g
Cremophor RH-40	0.41 g
Arlacel 186	0.29 g
Sodium taurocholate	0.26 g
Propylene glycol	0.46 g

15 For this experiment, the preconcentrate was diluted with an isotonic aqueous HEPES buffer rather than purified water. The resultant solution was spiked with radioactive active and perfused through isolated ileal lumen segment of known length and diameter. Loss of radioactivity from the luminal side and appearance of radioactivity in the mesenteric blood from the other side was monitored as an indicator of absorption.

 Experimental Details:

20 Young adult (275-300 g) male Sprague Dawley rats were used. The procedures were consistent with those reported by Winne et al., “In vivo studies of mucosal-serosal transfer in rat jejunum”, Naunyn-Schmeideberg’s Arch. Pharmacol., 329, 70 (1985).

25 Jugular vein cannulation: the animal was anesthetized using 2% halothane in 98% oxygen via a halothane vaporizer (Vapomatic, A.M. Bickford, Inc., NY). An opening in the jugular vein was made with a 21 ga needle and a jugular cannula consisting of a 4 cm segment of silastic tubing connected to polyethylene tubing was inserted in the jugular vein and secured with cyanoacrylate glue. For the donor rat, approximately 20 mL of blood was freshly collected in the presence of heparin (1,000 units) and the collected blood was infused at a rate of 0.2 mL/min through the jugular vein in the experimental rat to replenish blood
30 sampling.

 Intestine cannulation: after the animal was anesthetized, its body temperature was maintained at 37 °C using a heating pad. A vertical midline incision of approximately 3 cm was made through the skin to expose the small intestine. Approximately 6-10 cm segment of

1 ileum was located. Using electro-cautery, a small incision was made at the ends of the segment and the luminal contents were flushed with saline maintained at 37 °C. Two 1.5 cm notched pieces of Teflon tubing were inserted into the intestinal lumen at each incision and tightened using 4-0 silk. A warm isotonic buffer was passed through the intestine using a 50-
5 mL syringe. These Teflon cannula were used to perfuse the drug solution through the isolated intestinal segment using a syringe pump.

Mesenteric vein cannulation: the mesenteric vein draining blood from the resulting isolated mesenteric cascade venules was then cannulated using a 24 ga IV catheter and secured in place using 4-0 silk sutures. The cannula was then connected to a polyethylene
10 tubing 25 cm long where the blood was collected in a vial kept under the animal level. Blood samples were collected continuously over 60 min. The infusion of blood via the jugular vein was initiated to replenish blood loss. The animal was then killed by a lethal injection of Phenobarbital after completion of the experiment.

The experiment was performed twice using the compositions of the present invention
15 as the drug carrier, and twice using a commercial cyclosporin microemulsion formulation for comparison (NeOral®). For each formulation, the results of the two trials were averaged. The results are presented graphically in Figure 1.

Figure 1 shows the accumulated radioactivity ($\mu\text{Ci}/\text{cm}^2\mu\text{Ci}$) in mesenteric blood as a function of time, over the course of 60 minutes, for the pharmaceutical compositions of the
20 present invention (filled squares) and a commercial cyclosporin formulation (filled circles). As the Figure shows, the bioabsorption of the hydrophobic therapeutic agent exceeds that of the commercial formulation at the earliest measurement point, and continues to increase relative to the commercial formulation over the course of the measurement interval. At the final measurement point (60 min), the bioabsorption of the hydrophobic therapeutic agent
25 from the compositions of the present invention exceeds that of the commercial formulation by nearly 100%.

The present invention may be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments are to be considered in
30 all respects only as illustrative and not restrictive. The scope of the invention is, therefore, indicated by the appended claims rather than by the foregoing description. All changes which come within the meaning and range of equivalency of the claims are to be embraced within their scope.

What is claimed is:

- 1 1. A pharmaceutical composition comprising:
- (a) a hydrophobic therapeutic agent; and
- (b) a carrier,
- said carrier comprising:
- 5 (i) at least one hydrophilic surfactant; and
- (ii) at least one hydrophobic surfactant,
- said hydrophilic and hydrophobic surfactants being present in
- amounts such that upon mixing with an aqueous solution the carrier
- forms a clear aqueous dispersion of the hydrophilic and hydrophobic
- 10 surfactants containing the hydrophobic therapeutic agent,
- said composition being substantially free of triglycerides.
2. The pharmaceutical composition of claim 1, wherein the hydrophobic
- surfactant is present in an amount of less than about 200% by weight, relative to the amount
- of the hydrophilic surfactant.
- 15 3. The pharmaceutical composition of claim 2, wherein the hydrophobic
- surfactant is present in an amount of less than about 100% by weight, relative to the amount
- of the hydrophilic surfactant.
4. The pharmaceutical composition of claim 3, wherein the hydrophobic
- surfactant is present in an amount of less than about 60% by weight, relative to the amount of
- 20 the hydrophilic surfactant.
5. The pharmaceutical composition of claim 1, wherein the hydrophilic
- surfactant comprises at least one non-ionic hydrophilic surfactant having an HLB value
- greater than or equal to about 10.
6. The pharmaceutical composition of claim 1, wherein the hydrophilic
- 25 surfactant comprises at least one ionic surfactant.
7. The pharmaceutical composition of claim 5, which further comprises at least
- one ionic surfactant.
8. The pharmaceutical composition of claim 5, wherein the non-ionic surfactant
- is selected from the group consisting of alkylglucosides; alkylmaltosides;
- 30 alkylthioglucosides; lauryl macrogolglycerides; polyoxyethylene alkyl ethers;
- polyoxyethylene alkylphenols; polyethylene glycol fatty acids esters; polyethylene glycol
- glycerol fatty acid esters; polyoxyethylene sorbitan fatty acid esters; polyoxyethylene-
- polyoxypropylene block copolymers; polyglycerol fatty acid esters; polyoxyethylene

1 glycerides; polyoxyethylene sterols, derivatives, and analogues thereof; polyoxyethylene
vegetable oils; polyoxyethylene hydrogenated vegetable oils; reaction mixtures of polyols
and at least one member of the group consisting of fatty acids, glycerides, vegetable oils,
hydrogenated vegetable oils, and sterols; sugar esters, sugar ethers; sucroglycerides; and
5 mixtures thereof.

9. The pharmaceutical composition of claim 5, wherein the non-ionic hydrophilic
surfactant is selected from the group consisting of polyoxyethylene alkylethers; polyethylene
glycol fatty acids esters; polyethylene glycol glycerol fatty acid esters; polyoxyethylene
sorbitan fatty acid esters; polyoxyethylene-polyoxypropylene block copolymers; polyglycerol
10 fatty acid esters; polyoxyethylene glycerides; polyoxyethylene vegetable oils;
polyoxyethylene hydrogenated vegetable oils; reaction mixtures of polyols and at least one
member of the group consisting of fatty acids, glycerides, vegetable oils, hydrogenated
vegetable oils, and sterols; and mixtures thereof.

10. The pharmaceutical composition of claim 9, wherein the glyceride is a
15 monoglyceride, diglyceride, triglyceride, or a mixture thereof.

11. The pharmaceutical composition of claim 9, wherein the reaction mixture
comprises the transesterification products of a polyol and at least one member of the group
consisting of fatty acids, glycerides, vegetable oils, hydrogenated vegetable oils, and sterols.

12. The pharmaceutical composition of claim 9, wherein the polyol is glycerol,
20 ethylene glycol, polyethylene glycol, sorbitol, propylene glycol, pentaerythritol or a mixture
thereof.

13. The pharmaceutical composition of claim 5, wherein the hydrophilic
surfactant is PEG-10 laurate, PEG-12 laurate, PEG-20 laurate, PEG-32 laurate, PEG-32
dilaurate, PEG-12 oleate, PEG-15 oleate, PEG-20 oleate, PEG-20 dioleate, PEG-32 oleate,
25 PEG-200 oleate, PEG-400 oleate, PEG-15 stearate, PEG-32 distearate, PEG-40 stearate,
PEG-100 stearate, PEG-20 dilaurate, PEG-25 glyceryl trioleate, PEG-32 dioleate, PEG-20
glyceryl laurate, PEG-30 glyceryl laurate, PEG-20 glyceryl stearate, PEG-20 glyceryl oleate,
PEG-30 glyceryl oleate, PEG-30 glyceryl laurate, PEG-40 glyceryl laurate, PEG-40 palm
kernel oil, PEG-50 hydrogenated castor oil, PEG-40 castor oil, PEG-35 castor oil, PEG-60
30 castor oil, PEG-40 hydrogenated castor oil, PEG-60 hydrogenated castor oil, PEG-60 corn
oil, PEG-6 caprate/caprylate glycerides, PEG-8 caprate/caprylate glycerides, polyglyceryl-10
laurate, PEG-30 cholesterol, PEG-25 phyto sterol, PEG-30 soya sterol, PEG-20 trioleate,
PEG-40 sorbitan oleate, PEG-80 sorbitan laurate, polysorbate 20, polysorbate 80, POE-9

1 lauryl ether, POE-23 lauryl ether, POE-10 oleyl ether, POE-20 oleyl ether, POE-20 stearyl
ether, tocopheryl PEG-100 succinate, PEG-24 cholesterol, polyglyceryl-10 oleate, Tween 40,
Tween 60, sucrose monostearate, sucrose monolaurate, sucrose monopalmitate, PEG 10-100
nonyl phenol series, PEG 15-100 octyl phenol series, a poloxamer, or a mixture thereof.

5 14. The pharmaceutical composition of claim 5, wherein the hydrophilic
surfactant is PEG-20 laurate, PEG-20 oleate, PEG-35 castor oil, PEG-40 palm kernel oil,
PEG-40 hydrogenated castor oil, PEG-60 corn oil, PEG-25 glyceryl trioleate, polyglyceryl-10
laurate, PEG-6 caprate/caprylate glycerides, PEG-8 caprate/caprylate glycerides, PEG-30
cholesterol, polysorbate 20, polysorbate 80, POE-9 lauryl ether, POE-23 lauryl ether, POE-10
10 oleyl ether, PEG-24 cholesterol, sucrose monostearate, sucrose monolaurate, a poloxamer, or
a mixture thereof.

15 15. The pharmaceutical composition of claim 5, wherein the hydrophilic
surfactant is PEG-35 castor oil, PEG-40 hydrogenated castor oil, PEG-60 corn oil, PEG-25
glyceryl trioleate, PEG-6 caprate/caprylate glycerides, PEG-8 caprate/caprylate glycerides,
polysorbate 20, polysorbate 80, tocopheryl PEG-1000 succinate, PEG-24 cholesterol, a
poloxamer, or a mixture thereof.

20 16. The pharmaceutical composition of claim 6, wherein the ionic surfactant is
selected from the group consisting of alkyl ammonium salts; bile acids and salts, analogues,
and derivatives thereof; fatty acid derivatives of amino acids, oligopeptides, and
polypeptides; glyceride derivatives of amino acids, oligopeptides, and polypeptides; acyl
lactylates; mono-,diacetylated tartaric acid esters of mono-,diglycerides; succinylated
monoglycerides; citric acid esters of mono-,diglycerides; alginate salts; propylene glycol
alginate; lecithins and hydrogenated lecithins; lysolecithin and hydrogenated lysolecithins;
lysophospholipids and derivatives thereof; phospholipids and derivatives thereof; salts of
25 alkylsulfates; salts of fatty acids; sodium docusate; and mixtures thereof.

30 17. The pharmaceutical composition of claim 6, wherein the ionic surfactant is
selected from the group consisting of bile acids and salts, analogues, and derivatives thereof;
lecithins, lysolecithin, phospholipids, lysophospholipids and derivatives thereof; salts of
alkylsulfates; salts of fatty acids; sodium docusate; acyl lactylates; mono-,diacetylated tartaric
acid esters of mono-,diglycerides; succinylated monoglycerides; citric acid esters of mono-
diglycerides; and mixtures thereof.

18. The pharmaceutical composition of claim 6, wherein the ionic surfactant is
selected from the group consisting of lecithin, lysolecithin, phosphatidylcholine,

1 phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid, phosphatidylserine,
lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylglycerol,
lysophosphatidic acid, lysophosphatidylserine, PEG-phosphatidylethanolamine, PVP-
5 phosphatidylethanolamine, lactic esters of fatty acids, stearyl-2-lactylate, stearyl
lactylate, succinylated monoglycerides, mono/diacetylated tartaric acid esters of
mono/diglycerides, citric acid esters of mono/diglycerides, cholate, taurocholate,
glycocholate, deoxycholate, taurodeoxycholate, chenodeoxycholate, glycodeoxycholate,
glycochenodeoxycholate, taurochenodeoxycholate, ursodeoxycholate,
10 tauroursodeoxycholate, glyoursodeoxycholate, cholylsarcosine, N-methyl taurocholate,
caproate, caprylate, caprate, laurate, myristate, palmitate, oleate, ricinoleate, linoleate,
linolenate, stearate, lauryl sulfate, teracecyl sulfate, docusate, and salts and mixtures thereof.

19. The pharmaceutical composition of claim 6, wherein the ionic surfactant is
selected from the group consisting of lecithin, lysolecithin, phosphatidylcholine,
phosphatidylethanolamine, phosphatidylglycerol, lysophosphatidylcholine, PEG-
15 phosphatidylethanolamine, lactic esters of fatty acids, stearyl-2-lactylate, stearyl
lactylate, succinylated monoglycerides, mono/diacetylated tartaric acid esters of
mono/diglycerides, citric acid esters of mono/diglycerides, cholate, taurocholate,
glycocholate, deoxycholate, taurodeoxycholate, glycodeoxycholate, cholylsarcosine,
caproate, caprylate, caprate, laurate, oleate, lauryl sulfate, docusate, and salts and mixtures
20 thereof.

20. The pharmaceutical composition of claim 6, wherein the ionic surfactant is
selected from the group consisting of lecithin, lactic esters of fatty acids, stearyl-2-
lactylate, stearyl lactylate, succinylated monoglycerides, mono/diacetylated tartaric acid
esters of mono/diglycerides, citric acid esters of mono/diglycerides, taurocholate, caprylate,
25 caprate, oleate, lauryl sulfate, docusate, and salts and mixtures thereof.

21. The pharmaceutical composition of claim 1 wherein the hydrophobic
surfactant is a compound or mixture of compounds having an HLB value less than about 10.

22. The pharmaceutical composition of claim 21, wherein the hydrophobic
surfactant is selected from the group consisting of alcohols; polyoxyethylene alkylethers;
30 fatty acids; glycerol fatty acid esters; acetylated glycerol fatty acid esters; lower alcohol fatty
acids esters; polyethylene glycol fatty acids esters; polyethylene glycol glycerol fatty acid
esters; polypropylene glycol fatty acid esters; polyoxyethylene glycerides; lactic acid
derivatives of mono/diglycerides; propylene glycol diglycerides; sorbitan fatty acid esters;

1 polyoxyethylene sorbitan fatty acid esters; polyoxyethylene-polyoxypropylene block
copolymers; transesterified vegetable oils; sterols; sterol derivatives; sugar esters; sugar
ethers; sucroglycerides; polyoxyethylene vegetable oils; polyoxyethylene hydrogenated
vegetable oils; reaction mixtures of polyols and at least one member of the group consisting
5 of fatty acids, glycerides, vegetable oils, hydrogenated vegetable oils, and sterols; and
mixtures thereof.

23. The pharmaceutical composition of claim 21, wherein the hydrophobic
surfactant is selected from the group consisting of fatty acids; lower alcohol fatty acid esters;
polyethylene glycol glycerol fatty acid esters; polypropylene glycol fatty acid esters;
10 polyoxyethylene glycerides; glycerol fatty acid esters; acetylated glycerol fatty acid esters;
lactic acid derivatives of mono/diglycerides; sorbitan fatty acid esters; polyoxyethylene
sorbitan fatty acid esters; polyoxyethylene-polyoxypropylene block copolymers;
polyoxyethylene vegetable oils; polyoxyethylene hydrogenated vegetable oils; reaction
mixtures of polyols and at least one member of the group consisting of fatty acids, glycerides,
15 vegetable oils, hydrogenated vegetable oils, and sterols; and mixtures thereof.

24. The pharmaceutical composition of claim 21, wherein the hydrophobic
surfactant is selected from the group consisting of lower alcohol fatty acids esters;
polypropylene glycol fatty acid esters; propylene glycol fatty acid esters; glycerol fatty acid
esters; acetylated glycerol fatty acid esters; lactic acid derivatives of mono/diglycerides;
20 sorbitan fatty acid esters; polyoxyethylene vegetable oils; and mixtures thereof.

25. The pharmaceutical composition of claim 21, wherein the hydrophobic
surfactant is a glycerol fatty acid ester, an acetylated glycerol fatty acid ester, or a mixture
thereof.

26. The pharmaceutical composition of claim 25, wherein the glycerol fatty acid
25 ester is a monoglyceride, diglyceride, or a mixture thereof.

27. The pharmaceutical composition of claim 26, wherein the fatty acid of the
glycerol fatty acid ester is a C₆ to C₂₀ fatty acid or a mixture thereof.

28. The pharmaceutical composition of claim 21, wherein the hydrophobic
surfactant is a reaction mixture of a polyol and at least one member of the group consisting of
30 fatty acids, glycerides, vegetable oils, hydrogenated vegetable oils, and sterols.

29. The pharmaceutical composition of claim 28, wherein the reaction mixture is a
transesterification product of a polyol and at least one member of the group consisting of
fatty acids, glycerides, vegetable oils, hydrogenated vegetable oils, and sterols.

1 30. The pharmaceutical composition of claim 28, wherein the polyol is polyethylene glycol, sorbitol, propylene glycol, pentaerythritol or a mixture thereof.

 31. The pharmaceutical composition of claim 21, wherein the hydrophobic
surfactant is selected from the group consisting of myristic acid; oleic acid; lauric acid;
5 stearic acid; palmitic acid; PEG 1-4 stearate; PEG 2-4 oleate; PEG-4 dilaurate; PEG-4
dioleate; PEG-4 distearate; PEG-6 dioleate; PEG-6 distearate; PEG-8 dioleate; PEG 3-16
castor oil; PEG 5-10 hydrogenated castor oil; PEG 6-20 corn oil; PEG 6-20 almond oil; PEG-
6 olive oil; PEG-6 peanut oil; PEG-6 palm kernel oil; PEG-6 hydrogenated palm kernel oil;
PEG-4 capric/caprylic triglyceride, mono, di, tri, tetra esters of vegetable oil and sorbitol;
10 pentaerythrityl di, tetra stearate, isostearate, oleate, caprylate, or caprate; polyglyceryl 2-4
oleate, stearate, or isostearate; polyglyceryl 4-10 pentaoleate; polyglyceryl-3 dioleate;
polyglyceryl-6 dioleate; polyglyceryl-10 trioleate; polyglyceryl-3 distearate; propylene glycol
mono- or diesters of a C₆ to C₂₀ fatty acid; monoglycerides of a C₆ to C₂₀ fatty acid;
acetylated monoglycerides of C₆ to C₂₀ fatty acid; diglycerides of C₆ to C₂₀ fatty acids; lactic
15 acid derivatives of monoglycerides; lactic acid derivatives of diglycerides; cholesterol;
phytosterol; PEG 5-20 soya sterol; PEG-6 sorbitan tetra, hexastearate; PEG-6 sorbitan
tetraoleate; sorbitan monolaurate; sorbitan monopalmitate; sorbitan mono, trioleate; sorbitan
mono, tristearate; sorbitan monoisostearate; sorbitan sesquioleate; sorbitan sesquistearate;
PEG 2-5 oleyl ether; POE 2-4 lauryl ether; PEG-2 cetyl ether; PEG-2 stearyl ether; sucrose
20 distearate; sucrose dipalmitate; ethyl oleate; isopropyl myristate; isopropyl palmitate; ethyl
linoleate; isopropyl linoleate; poloxamers; and mixtures thereof.

 32. The pharmaceutical composition of claim 21, wherein the hydrophobic
surfactant is selected from the group consisting of oleic acid; lauric acid; glyceryl
monocaprinate; glyceryl monocaprylate; glyceryl monolaurate; glyceryl monooleate; glyceryl
25 dicaprinate; glyceryl dicaprylate; glyceryl dilaurate; glyceryl dioleate; acetylated
monoglycerides; propylene glycol oleate; propylene glycol laurate; polyglyceryl-3 oleate;
polyglyceryl-6 dioleate; PEG-6 corn oil; PEG-20 corn oil; PEG-20 almond oil; sorbitan
monooleate; sorbitan monolaurate; POE-4 lauryl ether; POE-3 oleyl ether; ethyl oleate;
poloxamers; and mixtures thereof.

30 33. The pharmaceutical composition of claim 1, wherein the clear aqueous
dispersion has a particle size distribution having an average particle size of less than about
100 nm.

1 34. The pharmaceutical composition of claim 33, wherein the clear aqueous dispersion has a particle size distribution having an average particle size of less than about 50 nm.

5 35. The pharmaceutical composition of claim 33, wherein the clear aqueous dispersion has a particle size distribution having an average particle size of less than about 20 nm.

 36. The pharmaceutical composition of claim 1, wherein the clear aqueous dispersion has an absorbance of less than about 0.1 at about 400 nm when the carrier is diluted with an aqueous solution in an aqueous solution to carrier ratio of 100:1 by weight.

10 37. The pharmaceutical composition of claim 36, wherein the absorbance is less than about 0.01.

 38. The pharmaceutical composition of claim 1, wherein the hydrophobic therapeutic agent has an intrinsic water solubility of less than about 1% by weight at 25 °C.

15 39. The pharmaceutical composition of claim 38, wherein the intrinsic water solubility is less than about 0.1% by weight at 25 °C.

 40. The pharmaceutical composition of claim 39, wherein the intrinsic water solubility is less than about 0.01% by weight at 25 °C.

 41. The pharmaceutical composition of claim 1, wherein the therapeutic agent is a drug, a vitamin, a nutritional supplement, a cosmeceutical, or a mixture thereof.

20 42. The pharmaceutical composition of claim 43, wherein the therapeutic agent is a polyfunctional hydrophobic drug, a lipophilic drug, a pharmaceutically acceptable salt, isomer or derivative thereof, or a mixture thereof.

 43. The pharmaceutical composition of claim 41, wherein the therapeutic agent is selected from the group consisting of analgesics, anti-inflammatory agents, anthelmintics, anti-arrhythmic agents, anti-bacterial agents, anti-viral agents, anti-coagulants, anti-depressants, anti-diabetics, anti-epileptics, anti-fungal agents, anti-gout agents, anti-hypertensive agents, anti-malarials, anti-migraine agents, anti-muscarinic agents, anti-neoplastic agents, erectile dysfunction improvement agents, immunosuppressants, anti-protozoal agents, anti-thyroid agents, anxiolytic agents, sedatives, hypnotics, neuroleptics, β -Blockers, cardiac inotropic agents, corticosteroids, diuretics, anti-parkinsonian agents, gastro-intestinal agents, histamine H₂-receptor antagonists, keratolytics, lipid regulating agents, anti-anginal agents, nutritional agents, opioid analgesics, sex hormones, stimulants, muscle relaxants, anti-osteoporosis agents, anti-obesity agents, cognition enhancers, anti-urinary

1 incontinence agents, nutritional oils, anti-benign prostate hypertrophy agents, essential fatty acids, non-essential fatty acids, and mixtures thereof.

44. The pharmaceutical composition of claim 41, wherein the therapeutic agent is
5 tramadol, celecoxib, etodolac, refocoxib, oxaprozin, leflunomide, diclofenac, nabumetone, ibuprofen, flurbiprofen, tetrahydrocannabinol, capsaicin, ketorolac, albendazole, ivermectin, amiodarone, zileuton, zafirlukast, albuterol, montelukast, azithromycin, ciprofloxacin, clarithromycin, dirithromycin, rifabutine, rifapentine, trovafloxacin, baclofen, ritanovir, saquinavir, nelfinavir, efavirenz, dicoumarol, tirofibrin, cilostazol, ticlidopine, clopidrogel, oprevelkin, paroxetine, sertraline, venlafaxine, bupropion, clomipramine, miglitol,
10 repaglinide, glymepride, pioglitazone, rosiglitazone, troglitazone, glyburide, glipizide, glibenclamide, carbamazepine, fosphenytoin, tiagabine, topiramate, lamotrigine, vigabatrin, amphotericin B, butenafine, terbinafine, itraconazole, flucanazole, miconazole, ketoconazole, metronidazole, griseofulvin, nitrofurantoin, spironolactone, lisinopril, benazepril, nifedipine, nilsolidipine, telmisartan, irbesartan, eprosartan, valsartan, candesartan, minoxidil, terazosin,
15 halofantrine, mefloquine, dihydroergotamine, ergotamine, frovatriptan, pizofetin, sumatriptan, zolmitriptan, naratriptan, rizatriptan, aminogluthemide, busulphan, cyclosporine, mitoxantrone, irinotecan, etoposide, teniposide, paclitaxel, tacrolimus, sirolimus, tamoxifen, camptothecin, topotecan, nilutamide, bicalutamide, pseudo-ephedrine, toremifene, atovaquone, metronidazole, furazolidone, paricalcitol, benzonatate, midazolam, zolpidem,
20 gabapentin, zopiclone, digoxin, beclomethasone, budesonide, betamethasone, prednisolone, cisapride, cimetidine, loperamide, famotidine, lansoprazole, rabeprazole, nizatidine, omeprazole, citrizine, cinnarizine, dexchlorpheniramine, loratadine, clemastine, fexofenadine, chlorpheniramine, acutretin, tazarotene, calcipotriene, calcitriol, targretin, ergocalciferol, cholecalciferol, isotretinoin, tretinoin, calcifediol, fenofibrate, probucol, gemfibrozil,
25 cerivastatin, pravastatin, simvastatin, fluvastatin, atorvastatin, tizanidine, dantrolene, isosorbide dinitrate, a carotene, dihydrotachysterol, vitamin A, vitamin D, vitamin E, vitamin K, an essential fatty acid source, codeine, fentanyl, methadone, nalbuphine, pentazocine, clomiphene, danazol, dihydro epiandrosterone, medroxyprogesterone, progesterone, rimexolone, megestrol acetate, osteradiol, finasteride, mifepristone, amphetamine, L-thyroxine,
30 tamsulosin, methoxsalen, tacrine, donepezil, raloxifene, vertoporphin, sibutramine, pyridostigmine, a pharmaceutically acceptable salt, isomer, or derivative thereof, or a mixture thereof.

1 45. The pharmaceutical composition of claim 1, wherein the hydrophobic
therapeutic agent is selected from the group consisting of tramadol, celecoxib, etodolac,
refocoxib, oxaprozin, leflunomide, diclofenac, nabumetone, ibuprofen, flurbiprofen,
5 tetrahydrocannabinol, capsaicin, ketorolac, albendazole, ivermectin, amiodarone, zileuton,
zafirlukast, albuterol, montelukast, azithromycin, ciprofloxacin, clarithromycin,
dirithromycin, rifabutine, rifapentine, trovafloxacin, baclofen, ritanovir, saquinavir,
nelfinavir, efavirenz, miglitol, repaglinide, glymepride, pioglitazone, rosigiltazone,
troglitazone, glyburide, glipizide, glibenclamide, carbamezepine, fosphenytion, tiagabine,
10 topiramate, lamotrigine, vigabatrin, amphotericin B, butenafine, terbinafine, itraconazole,
flucanazole, miconazole, ketoconazole, metronidazole, griseofulvin, nitrofurantoin,
spironolactone, halofantrine, mefloquine, dihydroergotamine, ergotamine, frovatriptan,
pizofetin, sumatriptan, zolmitriptan, naratriptan, rizatriptan, aminogluthemide, busulphan,
cyclosporine, mitoxantrone, irinotecan, etoposide, teniposide, paclitaxel, tacrolimus,
sirolimus, tamoxifen, camptothecin, topotecan, nilutamide, bicalutamide, pseudo-ephedrine,
15 toremifene, atovaquone, metronidazole, furzolidone, paricalcitol, benzonatate, midazolam,
zolpidem, gabapentin, zopiclone, digoxin, cisapride, cimetidine, loperamide, famotidine,
lanosprazole, rabeprazole, nizatidine, omeprazole, citrizine, cinnarizine, dexchlorpheniramine,
loratadine, clemastine, fexofenadine, chlorpheniramine, acutretin, tazarotene, calcipotriene,
calcitriol, targretin, ergocalciferol, cholecalciferol, isotretinoin, tretinoin, calcifediol,
20 fenofibrate, probucol, gemfibrozil, cerivastatin, pravastatin, simvastatin, fluvastatin,
atorvastatin, tizanidine, dantrolene, carotenes, dihyrotachysterol, vitamin A, vitamin D,
vitamin E, vitamin K, essential fatty acid sources, codeine, fentanyl, methadone, nalbuphine,
pentazocine, clomiphene, danazol, dihydro epiandrosterone, mmedoxyprogesterone,
progesterone, rimexolone, megestrol acetate, osteradiol, finasteride, mifepristone,
25 amphetamine, L-thyroxine, tamsulosin, methoxsalen, tacrine, donepezil, raloxifene,
vertopofin, sibutramine, pyridostigmine, pharmaceutically acceptable salts, isomers and
derivatives thereof, and mixtures thereof.

 46. The pharmaceutical composition of claim 1, wherein the therapeutic agent is
selected from the group consisting of tramadol, celecoxib, etodolac, refocoxib, oxaprozin,
30 leflunomide, diclofenac, nabumetone, ibuprofen, flurbiprofen, tetrahydrocannabinol,
capsaicin, ketorolac, ivermectin, amiodarone, zileuton, zafirlukast, albuterol, montelukast,
rifabutine, rifapentine, trovafloxacin, baclofen, ritanovir, saquinavir, nelfinavir, efavirenz,
miglitol, repaglinide, glymepride, pioglitazone, rosigiltazone, troglitazone, glyburide,

1 glipizide, glibenclamide, carbamazepine, fosphenytion, tiagabine, topiramate, lamotrigine,
vigabatrin, terbenafine, itraconazole, fluconazole, miconazole, ketoconazole, metronidazole,
nitrofurantoin, dihydroergotamine, ergotamine, frovatriptan, pizofetin, zolmitriptan, pseudo-
ephedrine, naratriptan, rizatriptan, aminogluthemide, busulphan, cyclosporine, mitoxantrone,
5 irinotecan, etoposide, teniposide, paclitaxel, tacrolimus, sirolimus, tamoxifen, camptothecin,
topotecan, nilutamide, bicalutamide, toremifene, atovaquone, metronidazole, furzolidone,
paricalcitol, benzonatate, cisapride, cimetidine, loperamide, famotidine, lansoprazole,
rabeprazole, nizatidine, omeprazole, citrizine, cinnarizine, dexchlorpheniramine, loratadine,
clemastine, fexofenadine, chlorpheniramine, acutretin, tazarotene, calcipotriene, calcitriol,
10 targretin, ergocalciferol, cholecalciferol, isotretinoin, tretinoin, calcifediol, fenofibrate,
probuco, simvastatin, atorvastatin, tizanidine, dantrolene, carotenes, dihydrocholesterol,
vitamin A, vitamin D, vitamin E, vitamin K, essential fatty acid sources, danazol, dihydro
epiandrosterone, medroxyprogesterone, progesterone, rimexolone, megestrol acetate,
osteradiol, finasteride, mefepiristone, raloxifene, L-thyroxine, tamsulosin, methoxsalen,
15 pharmaceutically acceptable salts, isomers and derivative thereof, and mixtures thereof.

47. The pharmaceutical composition of claim 1, wherein the hydrophobic
therapeutic agent is selected from the group consisting of sildenafil citrate, amlodipine,
tramadol, celecoxib, rofecoxib, oxaprozin, nabumetone, ibuprofen, terbenafine, itraconazole,
zileuton, zafirlukast, cisapride, fenofibrate, tizanidine, nizatidine, fexofenadine, loratadine,
20 famotidine, paricalcitol, atovaquone, nabumetone, tetrahydrocannabinol, megestrol acetate,
repaglinide, progesterone, rimexolone, cyclosporine, tacrolimus, sirolimus, teniposide,
paclitaxel, pseudo-ephedrine, troglitazone, rosiglitazone, finasteride, vitamin A, vitamin D,
vitamin E, pharmaceutically acceptable salts, isomers and derivatives thereof, and mixtures
thereof.

25 48. The pharmaceutical composition of claim 1, wherein the hydrophobic
therapeutic agent is progesterone or cyclosporin.

49. The pharmaceutical composition of claim 1, which further comprises a
solubilizer.

30 50. The pharmaceutical composition of claim 49, wherein the solubilizer is
selected from the group consisting of alcohols, polyols, amides, esters, propylene glycol
ethers and mixtures thereof.

51. The pharmaceutical composition of claim 50, wherein the alcohol or polyol is
selected from the group consisting of ethanol, isopropanol, butanol, benzyl alcohol, ethylene

1 glycol, propylene glycol, butanediols and isomers thereof, glycerol, pentaerythritol, sorbitol, mannitol, transcitol, dimethyl isosorbide, polyethylene glycol, polypropylene glycol, polyvinylalcohol, hydroxypropyl methylcellulose and other cellulose derivatives, cyclodextrins and cyclodextrin derivatives, and mixtures thereof.

5 52. The pharmaceutical composition of claim 50, wherein the amide is selected from the group consisting of 2-pyrrolidone, 2-piperidone, ϵ -caprolactam, N-alkylpyrrolidone, N-hydroxyalkylpyrrolidone, N-alkylpiperidone, N-alkylcaprolactam, dimethylacetamide, polyvinylpyrrolidone, and mixtures thereof.

10 53. The pharmaceutical composition of claim 50, wherein the ester is selected from the group consisting of ethyl propionate, tributylcitrate, acetyl triethylcitrate, acetyl tributyl citrate, triethylcitrate, ethyl oleate, ethyl caprylate, ethyl butyrate, triacetin, propylene glycol monoacetate, propylene glycol diacetate, ϵ -caprolactone and isomers thereof, δ -valerolactone and isomers thereof, β -butyrolactone and isomers thereof, and mixtures thereof.

15 54. The pharmaceutical composition of claim 49, wherein the solubilizer is selected from the group consisting of ethanol, isopropanol, butanol, benzyl alcohol, ethylene glycol, propylene glycol, butanediol and isomers thereof, glycerol, pentaerythritol, sorbitol, mannitol, transcitol, dimethyl isosorbide, polyethylene glycol, polypropylene glycol, polyvinylalcohol, hydroxypropyl methylcellulose and other cellulose derivatives, cyclodextrins, clodextrins and derivatives thereof, ethyl propionate, tributylcitrate, acetyl triethylcitrate, acetyl tributyl citrate, triethylcitrate, ethyl oleate, ethyl caprylate, ethyl butyrate, triacetin, propylene glycol diacetate, ϵ -caprolactone and isomers thereof, δ -valerolactone and isomers thereof, β -butyrolactone and isomers thereof, 2-pyrrolidone, 2-piperidone, ϵ -caprolactam, N-methylpyrrolidone, N-ethylpyrrolidone, N-hydroxyethyl
20 pyrrolidone, N-octylpyrrolidone, N-laurylpyrrolidone, dimethylacetamide, polyvinylpyrrolidone, glycofurol, methoxy PEG, and mixtures thereof.

25 55. The pharmaceutical composition of claim 49, wherein the solubilizer is selected from the group consisting of ethanol, isopropanol, benzyl alcohol, ethylene glycol, propylene glycol, 1,3-butanediol, glycerol, pentaerythritol, sorbitol, glycofurol, transcitol, dimethyl isosorbide, polyethylene glycol, polyvinylalcohol, hydroxypropyl methylcellulose, methylcellulose, ethylcellulose, hydroxypropylcyclodextrins, sulfobutyl ether derivatives of cyclodextrins, ethyl propionate, tributylcitrate, triethylcitrate, ethyl oleate, ethyl caprylate, triacetin, β -butyrolactone and isomers thereof, 2-pyrrolidone, N-methylpyrrolidone, N-
30

1 ethylpyrrolidone, N-hydroxyethylpyrrolidone, N-octylpyrrolidone, N-laurylpyrrolidone, dimethylacetamide, polyvinylpyrrolidone, and mixtures thereof.

5 56. The pharmaceutical composition of claim 49, wherein the solubilizer is triacetin, triethylcitrate, ethyl oleate, ethyl caprylate, dimethylacetamide, N-methylpyrrolidone, N-hydroxyethylpyrrolidone, polyvinylpyrrolidone, hydroxypropyl methylcellulose, hydroxypropyl cyclodextrins, ethanol, polyethylene glycol 200-600, glycofurol, transcitol, propylene glycol, dimethyl isosorbide, or a mixture thereof.

57. The pharmaceutical composition of claim 49, wherein the solubilizer is triacetin, ethanol, polyethylene glycol 400, glycofurol, propylene glycol or a mixture thereof.

10 58. The pharmaceutical composition of claim 49, wherein the solubilizer is present in the composition in an amount of about 400 % or less by weight, based on the total weight of the surfactants.

59. The pharmaceutical composition of claim 58, wherein the solubilizer is present in the composition in an amount of about 200 % or less by weight, based on the total weight of the surfactants.

60. The pharmaceutical composition of claim 59, wherein the solubilizer is present in the composition in an amount of about 100 % or less by weight, based on the total weight of the surfactants.

20 61. The pharmaceutical composition of claim 60, wherein the solubilizer is present in the composition in an amount of about 50 % or less by weight, based on the total weight of the surfactants.

62. The pharmaceutical composition of claim 61, wherein the solubilizer is present in the composition in an amount about 25 % or less by weight, based on the total weight of the surfactants.

25 63. The pharmaceutical composition of claim 1, which further comprises an antioxidant, a preservative, a chelating agent, a viscomodulator, a tonicifier, a flavorant, a colorant, an odorant, an opacifier, a suspending agent, a binder, or a mixture thereof.

64. The pharmaceutical composition of claim 1 in the form of a preconcentrate, a diluted preconcentrate, a semi-solid dispersion, a solid dispersion, or a sprayable solution.

30 65. A dosage form comprising a capsule filled with the pharmaceutical composition of claim 1.

66. A dosage form comprising a multiparticulate carrier coated with the pharmaceutical composition of claim 1.

1 67. A dosage form comprising the pharmaceutical composition of claim 1 formulated as a solution, a cream, a lotion, an ointment, a suppository, a spray, an aerosol, a paste or a gel.

5 68. The dosage form of claim 65, wherein the capsule is a hard gelatin capsule, a soft gelatin capsule, a starch capsule or an enteric coated capsule.

 69. The pharmaceutical composition of claim 1, which further comprises water or an aqueous buffer.

10 70. The pharmaceutical composition of claim 1, which further comprises an additional amount of a hydrophobic therapeutic agent, said additional amount not solubilized in the carrier.

 71. A pharmaceutical composition comprising:

- (a) at least one hydrophilic surfactant;
- (b) at least one hydrophobic surfactant; and
- (c) a hydrophobic therapeutic agent,

15 said pharmaceutical composition being in the form of a clear, aqueous dispersion which is substantially free of triglycerides.

 72. The pharmaceutical composition of claim 71, wherein the hydrophobic surfactant is present in an amount of less than about 200% by weight, relative to the amount of the hydrophilic surfactant.

20 73. The pharmaceutical composition of claim 72, wherein the hydrophobic surfactant is present in an amount of less than about 100% by weight, relative to the amount of the hydrophilic surfactant.

25 74. The pharmaceutical composition of claim 73, wherein the hydrophobic surfactant is present in an amount of less than about 60% by weight, relative to the amount of the hydrophilic surfactant.

 75. The pharmaceutical composition of claim 71, wherein the hydrophilic surfactant comprises at least one non-ionic hydrophilic surfactant having an HLB value greater than or equal to about 10.

30 76. The pharmaceutical composition of claim 71, wherein the hydrophilic surfactant comprises at least one ionic surfactant.

 77. The pharmaceutical composition of claim 75, which further comprises at least one ionic surfactant.

 78. The pharmaceutical composition of claim 75, wherein the non-ionic surfactant

1 is selected from the group consisting of alkylglucosides; alkylmaltosides;
alkylthioglucosides; lauryl macrogolglycerides; polyoxyethylene alkylethers;
polyoxyethylene alkylphenols; polyethylene glycol fatty acids esters; polyethylene glycol
glycerol fatty acid esters; polyoxyethylene sorbitan fatty acid esters; polyoxyethylene-
5 polyoxypropylene block copolymers; polyglycerol fatty acid esters; polyoxyethylene
glycerides; polyoxyethylene sterols, derivatives, and analogues thereof; polyoxyethylene
vegetable oils; polyoxyethylene hydrogenated vegetable oils; reaction mixtures of polyols
and at least one member of the group consisting of fatty acids, glycerides, vegetable oils,
hydrogenated vegetable oils, and sterols; sugar esters, sugar ethers; sucroglycerides; and
10 mixtures thereof.

79. The pharmaceutical composition of claim 75, wherein the non-ionic
hydrophilic surfactant is selected from the group consisting of polyoxyethylene alkylethers;
polyethylene glycol fatty acids esters; polyethylene glycol glycerol fatty acid esters;
polyoxyethylene sorbitan fatty acid esters; polyoxyethylene-polyoxypropylene block
15 copolymers; polyglycerol fatty acid esters; polyoxyethylene glycerides; polyoxyethylene
vegetable oils; polyoxyethylene hydrogenated vegetable oils; reaction mixtures of polyols
and at least one member of the group consisting of fatty acids, glycerides, vegetable oils,
hydrogenated vegetable oils, and sterols; and mixtures thereof.

80. The pharmaceutical composition of claim 79, wherein the glyceride is a
20 monoglyceride, diglyceride, triglyceride, or a mixture thereof.

81. The pharmaceutical composition of claim 79, wherein the reaction mixture
comprises the transesterification products of a polyol and at least one member of the group
consisting of fatty acids, glycerides, vegetable oils, hydrogenated vegetable oils, and sterols.

82. The pharmaceutical composition of claim 79, wherein the polyol is glycerol,
25 ethylene glycol, polyethylene glycol, sorbitol, propylene glycol, pentaerythritol or a mixture
thereof.

83. The pharmaceutical composition of claim 75, wherein the hydrophilic
surfactant is PEG-10 laurate, PEG-12 laurate, PEG-20 laurate, PEG-32 laurate, PEG-32
dilaurate, PEG-12 oleate, PEG-15 oleate, PEG-20 oleate, PEG-20 dioleate, PEG-32 oleate,
30 PEG-200 oleate, PEG-400 oleate, PEG-15 stearate, PEG-32 distearate, PEG-40 stearate,
PEG-100 stearate, PEG-20 dilaurate, PEG-25 glyceryl trioleate, PEG-32 dioleate, PEG-20
glyceryl laurate, PEG-30 glyceryl laurate, PEG-20 glyceryl stearate, PEG-20 glyceryl oleate,
PEG-30 glyceryl oleate, PEG-30 glyceryl laurate, PEG-40 glyceryl laurate, PEG-40 palm

1 kernel oil, PEG-50 hydrogenated castor oil, PEG-40 castor oil, PEG-35 castor oil, PEG-60
castor oil, PEG-40 hydrogenated castor oil, PEG-60 hydrogenated castor oil, PEG-60 corn
oil, PEG-6 caprate/caprylate glycerides, PEG-8 caprate/caprylate glycerides, polyglyceryl-10
laurate, PEG-30 cholesterol, PEG-25 phyto sterol, PEG-30 soya sterol, PEG-20 trioleate,
5 PEG-40 sorbitan oleate, PEG-80 sorbitan laurate, polysorbate 20, polysorbate 80, POE-9
lauryl ether, POE-23 lauryl ether, POE-10 oleyl ether, POE-20 oleyl ether, POE-20 stearyl
ether, tocopheryl PEG-100 succinate, PEG-24 cholesterol, polyglyceryl-10 oleate, Tween 40,
Tween 60, sucrose monostearate, sucrose monolaurate, sucrose monopalmitate, PEG 10-100
nonyl phenol series, PEG 15-100 octyl phenol series, a poloxamer, or a mixture thereof.

10 84. The pharmaceutical composition of claim 75, wherein the hydrophilic
surfactant is PEG-20 laurate, PEG-20 oleate, PEG-35 castor oil, PEG-40 palm kernel oil,
PEG-40 hydrogenated castor oil, PEG-60 corn oil, PEG-25 glyceryl trioleate, polyglyceryl-10
laurate, PEG-6 caprate/caprylate glycerides, PEG-8 caprate/caprylate glycerides, PEG-30
cholesterol, polysorbate 20, polysorbate 80, POE-9 lauryl ether, POE-23 lauryl ether, POE-10
15 oleyl ether, PEG-24 cholesterol, sucrose monostearate, sucrose monolaurate, a poloxamer, or
a mixture thereof.

20 85. The pharmaceutical composition of claim 75, wherein the hydrophilic
surfactant is PEG-35 castor oil, PEG-40 hydrogenated castor oil, PEG-60 corn oil, PEG-25
glyceryl trioleate, PEG-6 caprate/caprylate glycerides, PEG-8 caprate/caprylate glycerides,
polysorbate 20, polysorbate 80, tocopheryl PEG-1000 succinate, PEG-24 cholesterol, a
poloxamer, or a mixture thereof.

25 86. The pharmaceutical composition of claim 76, wherein the ionic surfactant is
selected from the group consisting of alkyl ammonium salts; bile acids and salts, analogues,
and derivatives thereof; fatty acid derivatives of amino acids, oligopeptides, and
polypeptides; glyceride derivatives of amino acids, oligopeptides, and polypeptides; acyl
lactylates; mono-,diacetylated tartaric acid esters of mono-,diglycerides; succinylated
monoglycerides; citric acid esters of mono-,diglycerides; alginate salts; propylene glycol
alginate; lecithins and hydrogenated lecithins; lysolecithin and hydrogenated lysolecithins;
lysophospholipids and derivatives thereof; phospholipids and derivatives thereof; salts of
30 alkylsulfates; salts of fatty acids; sodium docusate; and mixtures thereof.

87. The pharmaceutical composition of claim 76, wherein the ionic surfactant is
selected from the group consisting of bile acids and salts, analogues, and derivatives thereof;
lecithins, lysolecithin, phospholipids, lysophospholipids and derivatives thereof; salts of

1 alkylsulfates; salts of fatty acids; sodium docusate; acyl lactylates; mono-,diacetylated tartaric
acid esters of mono-,diglycerides; succinylated monoglycerides; citric acid esters of mono-
,diglycerides; and mixtures thereof.

5 88. The pharmaceutical composition of claim 76, wherein the ionic surfactant is
selected from the group consisting of lecithin, lysolecithin, phosphatidylcholine,
phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid, phosphatidylserine,
lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylglycerol,
lysophosphatidic acid, lysophosphatidylserine, PEG-phosphatidylethanolamine, PVP-
10 phosphatidylethanolamine, lactic esters of fatty acids, stearyl-2-lactylate, stearyl
lactylate, succinylated monoglycerides, mono/diacetylated tartaric acid esters of
mono/diglycerides, citric acid esters of mono/diglycerides, cholate, taurocholate,
glycocholate, deoxycholate, taurodeoxycholate, chenodeoxycholate, glycodeoxycholate,
glycochenodeoxycholate, taurochenodeoxycholate, ursodeoxycholate,
15 tauroursodeoxycholate, glyoursodeoxycholate, cholylsarcosine, N-methyl taurocholate,
caproate, caprylate, caprate, laurate, myristate, palmitate, oleate, ricinoleate, linoleate,
linolenate, stearate, lauryl sulfate, teracecyl sulfate, docusate, and salts and mixtures thereof.

20 89. The pharmaceutical composition of claim 76, wherein the ionic surfactant is
selected from the group consisting of lecithin, lysolecithin, phosphatidylcholine,
phosphatidylethanolamine, phosphatidylglycerol, lysophosphatidylcholine, PEG-
phosphatidylethanolamine, lactic esters of fatty acids, stearyl-2-lactylate, stearyl
lactylate, succinylated monoglycerides, mono/diacetylated tartaric acid esters of
mono/diglycerides, citric acid esters of mono/diglycerides, cholate, taurocholate,
glycocholate, deoxycholate, taurodeoxycholate, glycodeoxycholate, cholylsarcosine,
25 caproate, caprylate, caprate, laurate, oleate, lauryl sulfate, docusate, and salts and mixtures
thereof.

30 90. The pharmaceutical composition of claim 76, wherein the ionic surfactant is
selected from the group consisting of lecithin, lactic esters of fatty acids, stearyl-2-
lactylate, stearyl lactylate, succinylated monoglycerides, mono/diacetylated tartaric acid
esters of mono/diglycerides, citric acid esters of mono/diglycerides, taurocholate, caprylate,
caprate, oleate, lauryl sulfate, docusate, and salts and mixtures thereof.

91. The pharmaceutical composition of claim 71 wherein the hydrophobic
surfactant is a compound or mixture of compounds having an HLB value less than about 10.

1 92. The pharmaceutical composition of claim 91, wherein the hydrophobic
surfactant is selected from the group consisting of alcohols; polyoxyethylene alkylethers;
fatty acids; glycerol fatty acid esters; acetylated glycerol fatty acid esters; lower alcohol fatty
acids esters; polyethylene glycol fatty acids esters; polyethylene glycol glycerol fatty acid
5 esters; polypropylene glycol fatty acid esters; polyoxyethylene glycerides; lactic acid
derivatives of mono/diglycerides; propylene glycol diglycerides; sorbitan fatty acid esters;
polyoxyethylene sorbitan fatty acid esters; polyoxyethylene-polyoxypropylene block
copolymers; transesterified vegetable oils; sterols; sterol derivatives; sugar esters; sugar
ethers; sucroglycerides; polyoxyethylene vegetable oils; polyoxyethylene hydrogenated
10 vegetable oils; reaction mixtures of polyols and at least one member of the group consisting
of fatty acids, glycerides, vegetable oils, hydrogenated vegetable oils, and sterols; and
mixtures thereof.

 93. The pharmaceutical composition of claim 91, wherein the hydrophobic
surfactant is selected from the group consisting of fatty acids; lower alcohol fatty acid esters;
15 polyethylene glycol glycerol fatty acid esters; polypropylene glycol fatty acid esters;
polyoxyethylene glycerides; glycerol fatty acid esters; acetylated glycerol fatty acid esters;
lactic acid derivatives of mono/diglycerides; sorbitan fatty acid esters; polyoxyethylene
sorbitan fatty acid esters; polyoxyethylene-polyoxypropylene block copolymers;
polyoxyethylene vegetable oils; polyoxyethylene hydrogenated vegetable oils; reaction
20 mixtures of polyols and at least one member of the group consisting of fatty acids, glycerides,
vegetable oils, hydrogenated vegetable oils, and sterols; and mixtures thereof.

 94. The pharmaceutical composition of claim 91, wherein the hydrophobic
surfactant is selected from the group consisting of lower alcohol fatty acids esters;
polypropylene glycol fatty acid esters; propylene glycol fatty acid esters; glycerol fatty acid
25 esters; acetylated glycerol fatty acid esters; lactic acid derivatives of mono/diglycerides;
sorbitan fatty acid esters; polyoxyethylene vegetable oils; and mixtures thereof.

 95. The pharmaceutical composition of claim 91, wherein the hydrophobic
surfactant is a glycerol fatty acid ester, an acetylated glycerol fatty acid ester, or a mixture
thereof.

30 96. The pharmaceutical composition of claim 95, wherein the glycerol fatty acid
ester is a monoglyceride, diglyceride, or a mixture thereof.

 97. The pharmaceutical composition of claim 96, wherein the fatty acid of the
glycerol fatty acid ester is a C₆ to C₂₀ fatty acid or a mixture thereof.

1 98. The pharmaceutical composition of claim 91, wherein the hydrophobic surfactant is a reaction mixture of a polyol and at least one member of the group consisting of fatty acids, glycerides, vegetable oils, hydrogenated vegetable oils, and sterols.

5 99. The pharmaceutical composition of claim 98, wherein the reaction mixture is a transesterification product of a polyol and at least one member of the group consisting of fatty acids, glycerides, vegetable oils, hydrogenated vegetable oils, and sterols.

 100. The pharmaceutical composition of claim 98, wherein the polyol is polyethylene glycol, sorbitol, propylene glycol, pentaerythritol or a mixture thereof.

10 101. The pharmaceutical composition of claim 91, wherein the hydrophobic surfactant is selected from the group consisting of myristic acid; oleic acid; lauric acid; stearic acid; palmitic acid; PEG 1-4 stearate; PEG 2-4 oleate; PEG-4 dilaurate; PEG-4 dioleate; PEG-4 distearate; PEG-6 dioleate; PEG-6 distearate; PEG-8 dioleate; PEG 3-16 castor oil; PEG 5-10 hydrogenated castor oil; PEG 6-20 corn oil; PEG 6-20 almond oil; PEG-6 olive oil; PEG-6 peanut oil; PEG-6 palm kernel oil; PEG-6 hydrogenated palm kernel oil; 15 PEG-4 capric/caprylic triglyceride, mono, di, tri, tetra esters of vegetable oil and sorbitol; pentaerythrityl di, tetra stearate, isostearate, oleate, caprylate, or caprate; polyglyceryl 2-4 oleate, stearate, or isostearate; polyglyceryl 4-10 pentaoleate; polyglyceryl-3 dioleate; polyglyceryl-6 dioleate; polyglyceryl-10 trioleate; polyglyceryl-3 distearate; propylene glycol mono- or diesters of a C₆ to C₂₀ fatty acid; monoglycerides of a C₆ to C₂₀ fatty acid; 20 acetylated monoglycerides of C₆ to C₂₀ fatty acid; diglycerides of C₆ to C₂₀ fatty acids; lactic acid derivatives of monoglycerides; lactic acid derivatives of diglycerides; cholesterol; phytosterol; PEG 5-20 soya sterol; PEG-6 sorbitan tetra, hexastearate; PEG-6 sorbitan tetraoleate; sorbitan monolaurate; sorbitan monopalmitate; sorbitan mono, trioleate; sorbitan mono, tristearate; sorbitan monoisostearate; sorbitan sesquioleate; sorbitan sesquisteate; 25 PEG 2-5 oleyl ether; POE 2-4 lauryl ether; PEG-2 cetyl ether; PEG-2 stearyl ether; sucrose distearate; sucrose dipalmitate; ethyl oleate; isopropyl myristate; isopropyl palmitate; ethyl linoleate; isopropyl linoleate; poloxamers; and mixtures thereof.

30 102. The pharmaceutical composition of claim 91, wherein the hydrophobic surfactant is selected from the group consisting of oleic acid; lauric acid; glyceryl monocaprate; glyceryl monocaprylate; glyceryl monolaurate; glyceryl monooleate; glyceryl dicaprate; glyceryl dicaprylate; glyceryl dilaurate; glyceryl dioleate; acetylated monoglycerides; propylene glycol oleate; propylene glycol laurate; polyglyceryl-3 oleate; polyglyceryl-6 dioleate; PEG-6 corn oil; PEG-20 corn oil; PEG-20 almond oil; sorbitan

1 monooleate; sorbitan monolaurate; POE-4 lauryl ether; POE-3 oleyl ether; ethyl oleate; poloxamers; and mixtures thereof.

103. The pharmaceutical composition of claim 71, wherein the clear aqueous dispersion has a particle size distribution having an average particle size of less than about
5 100 nm.

104. The pharmaceutical composition of claim 103, wherein the clear aqueous dispersion has a particle size distribution having an average particle size of less than about 50 nm.

105. The pharmaceutical composition of claim 103, wherein the clear aqueous
10 dispersion has a particle size distribution having an average particle size of less than about 20 nm.

106. The pharmaceutical composition of claim 71, wherein the clear aqueous dispersion has an absorbance of less than about 0.1 at 400 nm when the ratio of the weight of water to the total weight of the hydrophilic surfactant, the hydrophobic surfactant and the
15 therapeutic agent is 100:1.

107. The pharmaceutical composition of claim 106, wherein the absorbance is less than about 0.01.

108. The pharmaceutical composition of claim 71, wherein the hydrophobic therapeutic agent has an intrinsic water solubility of less than about 1% by weight at 25 °C.

20 109. The pharmaceutical composition of claim 108, wherein the intrinsic water solubility is less than about 0.1% by weight at 25 °C.

110. The pharmaceutical composition of claim 109, wherein the intrinsic water solubility is less than about 0.01% by weight at 25 °C.

25 111. The pharmaceutical composition of claim 71, wherein the therapeutic agent is a drug, a vitamin, a nutritional supplement, a cosmeceutical, or a mixture thereof.

112. The pharmaceutical composition of claim 111, wherein the therapeutic agent is a polyfunctional hydrophobic drug, a lipophilic drug, a pharmaceutically acceptable salt, isomer or derivative thereof, or a mixture thereof.

30 113. The pharmaceutical composition of claim 111, wherein the therapeutic agent is selected from the group consisting of analgesics, anti-inflammatory agents, anthelmintics, anti-arrhythmic agents, anti-bacterial agents, anti-viral agents, anti-coagulants, anti-depressants, anti-diabetics, anti-epileptics, anti-fungal agents, anti-gout agents, anti-hypertensive agents, anti-malarials, anti-migraine agents, anti-muscarinic agents, anti-

1 neoplastic agents, erectile dysfunction improvement agents, immunosuppressants, anti-
protozoal agents, anti-thyroid agents, anxiolytic agents, sedatives, hypnotics, neuroleptics, β -
Blockers, cardiac inotropic agents, corticosteroids, diuretics, anti-parkinsonian agents, gastro-
intestinal agents, histamine H₂-receptor antagonists, keratolytics, lipid regulating agents, anti-
5 anginal agents, nutritional agents, opioid analgesics, sex hormones, stimulants, muscle
relaxants, anti-osteoporosis agents, anti-obesity agents, cognition enhancers, anti-urinary
incontinence agents, nutritional oils, anti-benign prostate hypertrophy agents, essential fatty
acids, non-essential fatty acids, and mixtures thereof.

10 114. The pharmaceutical composition of claim 111, wherein the therapeutic agent
is tramadol, celecoxib, etodolac, refocoxib, oxaprozin, leflunomide, diclofenac, nabumetone,
ibuprofen, flurbiprofen, tetrahydrocannabinol, capsaicin, ketorolac, albendazole, ivermectin,
amiodarone, zileuton, zafirlukast, albuterol, montelukast, azithromycin, ciprofloxacin,
clarithromycin, dirithromycin, rifabutine, rifapentine, trovafloxacin, baclofen, ritanovir,
15 saquinavir, nelfinavir, efavirenz, dicoumarol, tirofibrin, cilostazol, ticlidopine, clopidrogel,
oprelvekin, paroxetine, sertraline, venlafaxine, bupropion, clomipramine, miglitol,
repaglinide, glymepride, pioglitazone, rosiglitazone, troglitazone, glyburide, glipizide,
glibenclamide, carbamazepine, fosphenytoin, tiagabine, topiramate, lamotrigine, vigabatrin,
amphotericin B, butenafine, terbinafine, itraconazole, fluconazole, miconazole, ketoconazole,
metronidazole, griseofulvin, nitrofurantoin, spironolactone, lisinopril, benazepril, nifedipine,
20 nilsolidipine, telmisartan, irbesartan, eprosartan, valsartan, candesartan, minoxidil, terazosin,
halofantrine, mefloquine, dihydroergotamine, ergotamine, frovatriptan, pizofetin,
sumatriptan, zolmitriptan, naratriptan, rizatriptan, aminogluthemide, busulphan, cyclosporine,
mitoxantrone, irinotecan, etoposide, teniposide, paclitaxel, tacrolimus, sirolimus, tamoxifen,
camptothecin, topotecan, nilutamide, bicalutamide, ephedrine, toremifene, atovaquone,
25 metronidazole, furazolidone, paricalcitol, benzonatate, midazolam, zolpidem, gabapentin,
zopiclone, digoxin, beclomethasone, budesonide, betamethasone, prednisolone, cisapride,
cimetidine, loperamide, famotidine, lansoprazole, rabeprazole, nizatidine, omeprazole,
citrizine, cinnarizine, dexchlorpheniramine, loratadine, clemastine, fexofenadine,
chlorpheniramine, acutretin, tazarotene, calcipotriene, calcitriol, targretin, ergocalciferol,
30 cholecalciferol, isotretinoin, tretinoin, calcifediol, fenofibrate, probucol, gemfibrozil,
cerivastatin, pravastatin, simvastatin, fluvastatin, atorvastatin, tizanidine, dantrolene,
isosorbide dinitrate, a carotene, dihydrotachysterol, vitamin A, vitamin D, vitamin E, vitamin
K, an essential fatty acid source, codeine, fentanyl, methadone, nalbuphine, pentazocine,

1 clomiphene, danazol, dihydro epiandrosterone, medroxyprogesterone, progesterone,
rimexolone, megestrol acetate, osteradiol, finasteride, mifepristone, amphetamine, L-
thyroxine, tamsulosin, methoxsalen, tacrine, donepezil, raloxifene, vertoporphin, sibutramine,
pyridostigmine, a pharmaceutically acceptable salt, isomer, or derivative thereof, or a mixture
5 thereof.

115. The pharmaceutical composition of claim 71, wherein the hydrophobic
therapeutic agent is selected from the group consisting of tramadol, celecoxib, etodolac,
refocoxib, oxaprozin, leflunomide, diclofenac, nabumetone, ibuprofen, flurbiprofen,
tetrahydrocannabinol, capsaicin, ketorolac, albendazole, ivermectin, amiodarone, zileuton,
10 zafirlukast, albuterol, montelukast, azithromycin, ciprofloxacin, clarithromycin,
dirithromycin, rifabutin, rifapentine, trovafloxacin, baclofen, ritanovir, saquinavir,
nelfinavir, efavirenz, miglitol, repaglinide, glymepride, pioglitazone, rosiglitazone,
troglitazone, glyburide, glipizide, glibenclamide, carbamazepine, fosphenytoin, tiagabine,
topiramate, lamotrigine, vigabatrin, amphotericin B, butenafine, terbinafine, itraconazole,
15 flucanazole, miconazole, ketoconazole, metronidazole, griseofulvin, nitrofurantoin,
spironolactone, halofantrine, mefloquine, dihydroergotamine, ergotamine, frovatriptan,
pizofetin, sumatriptan, zolmitriptan, naratriptan, rizatriptan, aminogluthemide, busulphan,
cyclosporine, mitoxantrone, irinotecan, etoposide, teniposide, paclitaxel, tacrolimus,
sirolimus, tamoxifen, camptothecin, topotecan, nilutamide, bicalutamide, pseudo-ephedrine,
20 toremifene, atovaquone, metronidazole, furzolidone, paricalcitol, benzonatate, midazolam,
zolpidem, gabapentin, zopiclone, digoxin, cisapride, cimetidine, loperamide, famotidine,
lanosprazole, rabeprazole, nizatidine, omeprazole, citrizine, cinnarizine, dexchlorpheniramine,
loratadine, clemastine, fexofenadine, chlorpheniramine, acutretin, tazarotene, calcipotriene,
calcitriol, targretin, ergocalciferol, cholecalciferol, isotretinoin, tretinoin, calcifediol,
25 fenofibrate, probucol, gemfibrozil, cerivastatin, pravastatin, simvastatin, fluvastatin,
atorvastatin, tizanidine, dantrolene, carotenes, dihydroxycholesterol, vitamin A, vitamin D,
vitamin E, vitamin K, essential fatty acid sources, codeine, fentanyl, methadone, nalbuphine,
pentazocine, clomiphene, danazol, dihydro epiandrosterone, medroxyprogesterone,
progesterone, rimexolone, megestrol acetate, osteradiol, finasteride, mifepristone,
30 amphetamine, L-thyroxine, tamsulosin, methoxsalen, tacrine, donepezil, raloxifene,
vertoporphin, sibutramine, pyridostigmine, pharmaceutically acceptable salts, isomers and
derivatives thereof, and mixtures thereof.

1 116. The pharmaceutical composition of claim 71, wherein the therapeutic agent is
selected from the group consisting of tramadol, celecoxib, etodolac, refocoxib, oxaprozin,
leflunomide, diclofenac, nabumetone, ibuprofen, flurbiprofen, tetrahydrocannabinol,
capsaicin, ketorolac, ivermectin, amiodarone, zileuton, zafirlukast, albuterol, montelukast,
5 rifabutine, rifapentine, trovafloxacin, baclofen, ritanovir, saquinavir, nelfinavir, efavirenz,
miglitol, repaglinide, glymepride, pioglitazone, rosiglitazone, troglitazone, glyburide,
glipizide, glibenclamide, carbamezepine, fosphenytion, tiagabine, topiramate, lamotrigine,
vigabatrin, terbenafine, itraconazole, flucanazole, miconazole, ketoconazole, metronidazole,
nitrofurantoin, dihydroergotamine, ergotamine, frovatriptan, pizofetin, zolmitriptan, pseudo-
10 ephedrine, naratriptan, rizatriptan, aminogluthemide, busulphan, cyclosporine, mitoxantrone,
irinotecan, etoposide, teniposide, paclitaxel, tacrolimus, sirolimus, tamoxifen, camptothecin,
topotecan, nilutamide, bicalutamide, toremifene, atovaquone, metronidazole, furzolidone,
paricalcitol, benzonatate, cisapride, cimetidine, loperamide, famotidine, lansoprazole,
rabeprazole, nizatidine, omeprazole, citrizine, cinnarizine, dexchlorpheniramine, loratadine,
15 clemastine, fexofenadine, chlorpheniramine, acutretin, tazarotene, calcipotriene, calcitriol,
targretin, ergocalciferol, cholecalciferol, isotretinoin, tretinoin, calcifediol, fenofibrate,
probucol, simvastatin, atorvastatin, tizanidine, dantrolene, carotenes, dihydroxycholesterol,
vitamin A, vitamin D, vitamin E, vitamin K, essential fatty acid sources, danazol, dihydro
epiandrosterone, medroxyprogesterone, progesterone, rimexolone, megestrol acetate,
20 osteradiol, finasteride, mifepristone, raloxifene, L-thyroxine, tamsulosin, methoxsalen,
pharmaceutically acceptable salts, isomers and derivative thereof, and mixtures thereof.

 117. The pharmaceutical composition of claim 71, wherein the hydrophobic
therapeutic agent is selected from the group consisting of sildenafil citrate, amlodipine,
tramadol, celecoxib, refocoxib, oxaprozin, nabumetone, ibuprofen, terbenafine, itraconazole,
25 zileuton, zafirlukast, cisapride, fenofibrate, tizanidine, nizatidine, fexofenadine, loratadine,
famotidine, paricalcitol, atovaquone, nabumetone, tetrahydrocannabinol, megestrol acetate,
repaglinide, progesterone, rimexolone, cyclosporine, tacrolimus, sirolimus, teniposide,
paclitaxel, pseudo-ephedrine, troglitazone, rosiglitazone, finasteride, vitamin A, vitamin D,
vitamin E, pharmaceutically acceptable salts, isomers and derivatives thereof, and mixtures
30 thereof.

 118. The pharmaceutical composition of claim 71, wherein the hydrophobic
therapeutic agent is progesterone or cyclosporin.

1 119. The pharmaceutical composition of claim 71, which further comprises a solubilizer.

 120. The pharmaceutical composition of claim 119, wherein the solubilizer is selected from the group consisting of alcohols, polyols, amides, esters, polyethylene glycol
5 ethers and mixtures thereof.

 121. The pharmaceutical composition of claim 120, wherein the alcohol or polyol is selected from the group consisting of ethanol, isopropanol, butanol, benzyl alcohol, ethylene glycol, propylene glycol, butanediols and isomers thereof, glycerol, pentaerythritol, sorbitol, mannitol, transcitol, dimethyl isosorbide, polyethylene glycol, polypropylene glycol,
10 polyvinylalcohol, hydroxypropyl methylcellulose and other cellulose derivatives, cyclodextrins and cyclodextrin derivatives, and mixtures thereof.

 122. The pharmaceutical composition of claim 120, wherein the amide is selected from the group consisting of 2-pyrrolidone, 2-piperidone, ϵ -caprolactam, N-alkylpyrrolidone, N-hydroxyalkylpyrrolidone, N-alkylpiperidone, N-alkylcaprolactam, dimethylacetamide,
15 polyvinylpyrrolidone, and mixtures thereof.

 123. The pharmaceutical composition of claim 120, wherein the ester is selected from the group consisting of ethyl propionate, tributylcitrate, acetyl triethylcitrate, acetyl tributyl citrate, triethylcitrate, ethyl oleate, ethyl caprylate, ethyl butyrate, triacetin, propylene glycol monoacetate, propylene glycol diacetate, ϵ -caprolactone and isomers thereof, δ -
20 valerolactone and isomers thereof, β -butyrolactone and isomers thereof, and mixtures thereof.

 124. The pharmaceutical composition of claim 119, wherein the solubilizer is selected from the group consisting of ethanol, isopropanol, butanol, benzyl alcohol, ethylene glycol, propylene glycol, butanediol and isomers thereof, glycerol, pentaerythritol, sorbitol, mannitol, transcitol, dimethyl isosorbide, polyethylene glycol, polypropylene glycol,
25 polyvinylalcohol, hydroxypropyl methylcellulose and other cellulose derivatives, cyclodextrins, clodextrins and derivatives thereof, ethyl propionate, tributylcitrate, acetyl triethylcitrate, acetyl tributyl citrate, triethylcitrate, ethyl oleate, ethyl caprylate, ethyl butyrate, triacetin, propylene glycol diacetate, ϵ -caprolactone and isomers thereof, δ -
30 valerolactone and isomers thereof, β -butyrolactone and isomers thereof, 2-pyrrolidone, 2-piperidone, ϵ -caprolactam, N-methylpyrrolidone, N-ethylpyrrolidone, N-hydroxyethyl pyrrolidone, N-octylpyrrolidone, N-laurylpyrrolidone, dimethylacetamide, polyvinylpyrrolidone, glycofurol, methoxy PEG, and mixtures thereof.

1 125. The pharmaceutical composition of claim 119, wherein the solubilizer is
selected from the group consisting of ethanol, isopropanol, benzyl alcohol, ethylene glycol,
propylene glycol, 1,3-butanediol, glycerol, pentaerythritol, sorbitol, glycofurol, transcitol,
5 dimethyl isosorbide, polyethylene glycol, polyvinylalcohol, hydroxypropyl methylcellulose,
methylcellulose, ethylcellulose, hydroxypropylcyclodextrins, sulfobutyl ether derivatives of
cyclodextrins, ethyl propionate, tributylcitrate, triethylcitrate, ethyl oleate, ethyl caprylate,
triacetin, β -butyrolactone and isomers thereof, 2-pyrrolidone, N-methylpyrrolidone, N-
ethylpyrrolidone, N-hydroxyethylpyrrolidone, N-octylpyrrolidone, N-laurylpyrrolidone,
10 dimethylacetamide, polyvinylpyrrolidone, and mixtures thereof.

10 126. The pharmaceutical composition of claim 119, wherein the solubilizer is
triacetin, triethylcitrate, ethyl oleate, ethyl caprylate, dimethylacetamide, N-
methylpyrrolidone, N-hydroxyethylpyrrolidone, polyvinylpyrrolidone, hydroxypropyl
methylcellulose, hydroxypropyl cyclodextrins, ethanol, polyethylene glycol 200-600,
glycofurol, transcitol, propylene glycol, dimethyl isosorbide, or a mixture thereof.

15 127. The pharmaceutical composition of claim 119, wherein the solubilizer is
triacetin, ethanol, polyethylene glycol 400, glycofurol, propylene glycol or a mixture thereof.

 128. The pharmaceutical composition of claim 119, wherein the solubilizer is
present in the composition in an amount of about 400 % or less by weight, based on the total
weight of the surfactants.

20 129. The pharmaceutical composition of claim 128, wherein the solubilizer is
present in the composition in an amount of about 200 % or less by weight, based on the total
weight of the surfactants.

 130. The pharmaceutical composition of claim 129, wherein the solubilizer is
present in the composition in an amount of about 100 % or less by weight, based on the total
25 weight of the surfactants.

 131. The pharmaceutical composition of claim 130, wherein the solubilizer is
present in the composition in an amount of about 50 % or less by weight, based on the total
weight of the surfactants.

30 132. The pharmaceutical composition of claim 131, wherein the solubilizer is
present in the composition in an amount about 25 % or less by weight, based on the total
weight of the surfactants.

1 133. The pharmaceutical composition of claim 71, which further comprises an antioxidant, a preservative, a chelating agent, a viscomodulator, a tonicifier, a flavorant, a colorant, an odorant, an opacifier or a mixture thereof.

5 134. The pharmaceutical composition of claim 71, which further comprises an additional amount of a hydrophobic therapeutic agent, said additional amount not solubilized in the carrier.

 135. A pharmaceutical composition comprising:

 (a) a carrier,

 said carrier comprising:

10 (i) at least one hydrophilic surfactant; and

 (ii) at least one hydrophobic surfactant,

 said hydrophilic and hydrophobic surfactants being present in amounts such that upon mixing with an aqueous solution the carrier forms a clear aqueous dispersion of the hydrophilic and hydrophobic surfactants;

15 (b) a first amount of a hydrophobic therapeutic agent, said first amount being solubilized in the carrier; and

 (c) a second amount of a hydrophobic therapeutic agent, said second amount not solubilized in the clear aqueous dispersion, said composition being substantially free of triglycerides.

20 136. A method of treating an animal with a hydrophobic therapeutic agent, the method comprising:

 providing a dosage form of a pharmaceutical composition comprising:

 a hydrophobic therapeutic agent; and

25 a carrier,

 said carrier comprising:

 at least one hydrophilic surfactant; and

 at least one hydrophobic surfactant,

30 said hydrophilic and hydrophobic surfactants being present in amounts such that upon mixing with an aqueous solution the carrier forms a clear aqueous dispersion of the hydrophilic and hydrophobic surfactants containing the hydrophobic therapeutic agent,

 said composition being substantially free of triglycerides; and

1 administering said dosage form to said animal.

137. The method of claim 136, wherein the dosage form is a capsule, a cream, a lotion, an ointment, a suppository, a paste or a gel.

5 138. The method of claim 136, wherein the dosage form is administered by an oral, parenteral, topical, transdermal, ocular, pulmonary, vaginal, rectal or transmucosal route.

139. The method of claim 136, wherein the animal is a mammal.

140. The method of claim 139, wherein the mammal is a human.

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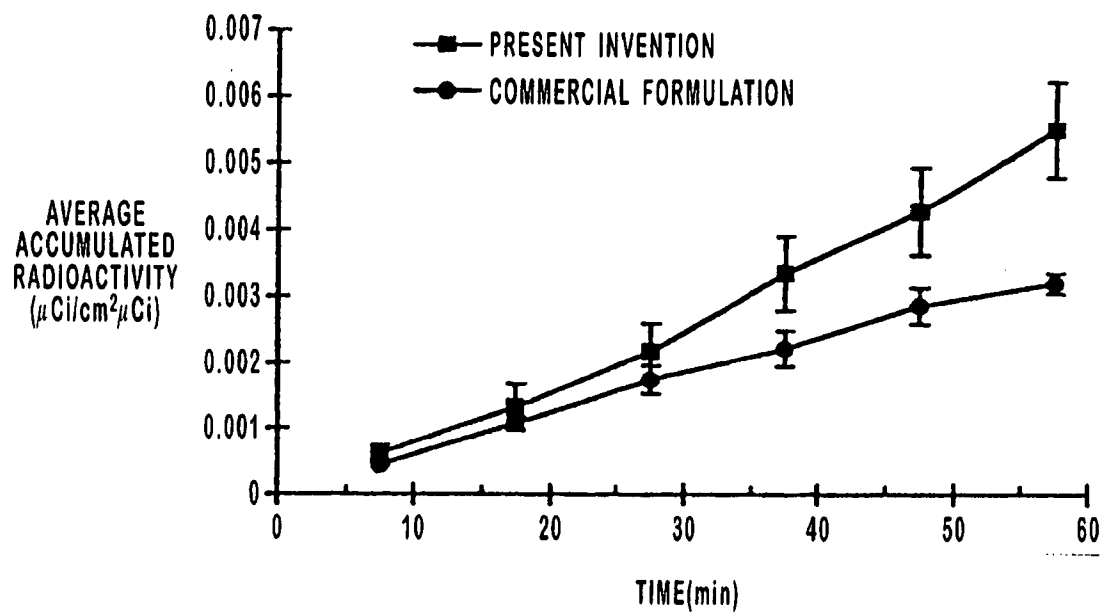


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/00165

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 9/127, 9/107, 38/13

US CL : 424/450, 455, 456; 514/937, 938

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450, 455, 456; 514/937, 938

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
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WEST**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,719,239 A (MULLER et al) 12 January 1988, see entire document.	1-140
Y	US 4,944,949 A (STORY et al) 31 July 1990, see entire document.	1-140
X	US 4,572,915 A (CROOKS) 25 February 1986, see entire document.	1-140
X	US 4,727,109 A (SCHMIDT et al) 23 February 1988, see entire document.	1-140

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search

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(21) Numéro de la demande internationale: PCT/FR97/02084 (22) Date de dépôt international: 19 novembre 1997 (19.11.97) (30) Données relatives à la priorité: 96/15046 6 décembre 1996 (06.12.96) FR (71) Déposant (pour tous les Etats désignés sauf US): L'OREAL [FR/FR]; 14, rue Royale, F-75008 Paris (FR). (72) Inventeurs; et (75) Inventeurs/Déposants (US seulement): DURANTON, Albert [FR/FR]; 7, rue Jacques Kablé, F-75018 Paris (FR). PRUCHE, Francis [FR/FR]; 5, Villa Moncalm, F-75018 Paris (FR). (74) Mandataire: TEZIER HERMAN, Béatrice; L'Oréal - DPI, 90, rue du Général Roguet, F-92583 Clichy Cedex (FR).		(81) Etats désignés: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, brevet ARIPO (GH, KE, LS, MW, SD, SZ, UG, ZW), brevet eurasien (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), brevet européen (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), brevet OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Publiée <i>Avec rapport de recherche internationale. Avant l'expiration du délai prévu pour la modification des revendications, sera republiée si de telles modifications sont reçues.</i>
(54) Title: USE OF PARACETAMOL AS DEPIGMENTING AGENT (54) Titre: UTILISATION DU PARACETAMOL COMME AGENT DEPIGMENTANT (57) Abstract <p>The invention concerns the use of paracetamol in a composition as depigmenting and/or bleaching agent for human skin, hairs and/or hair, and a depigmenting composition containing paracetamol. It also concerns a method for depigmenting and/or bleaching the skin, hairs and/or hair, which consists in applying on human skin, hairs and/or hair a composition containing paracetamol.</p> (57) Abrégé <p>L'invention se rapporte à l'utilisation du paracétamol dans une composition comme agent dépigmentant et/ou blanchissant de la peau humaine, des poils et/ou des cheveux, ainsi qu'à une composition dépigmentante contenant du paracétamol. Elle se rapporte également à un procédé de dépigmentation et/ou de blanchissement de la peau, des poils et/ou des cheveux, consistant à appliquer sur la peau humaine, les poils et/ou les cheveux une composition comprenant le paracétamol.</p>		

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Codes utilisés pour identifier les Etats parties au PCT, sur les pages de couverture des brochures publiant des demandes internationales en vertu du PCT.

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Utilisation du paracétamol comme agent dépigmentant

La présente invention se rapporte à l'utilisation du paracétamol comme agent
dépigmentant ou blanchissant dans une composition cosmétique et/ou
5 dermatologique, ainsi qu'à une composition dépigmentante et/ou blanchissante
contenant le paracétamol.

La couleur de la peau humaine est fonction de différents facteurs et notamment
des saisons de l'année, de la race et du sexe, et elle est principalement
10 déterminée par la nature et la concentration de mélanine produite par les
mélanocytes. Les mélanocytes sont les cellules spécialisées qui par
l'intermédiaire d'organelles particuliers, les mélanosomes, synthétisent la
mélanine. En outre, à différentes périodes de leur vie, certaines personnes
voient apparaître sur la peau et plus spécialement sur les mains, des taches
15 plus foncées et/ou plus colorées, conférant à la peau une hétérogénéité. Ces
taches sont dues aussi à une concentration importante de mélanine dans les
kératinocytes situés à la surface de la peau.

De la même manière, la couleur des poils et des cheveux est due à la
20 mélanine, lorsque les poils ou les cheveux sont foncés, certaines personnes
désirent voir ceux-ci plus claires. Ceci est particulièrement intéressant pour les
poils qui sont moins visibles lorsqu'ils sont clairs que lorsqu'ils sont foncés.

Le mécanisme de formation de la pigmentation de la peau, des poils et des
25 cheveux, c'est-à-dire de la formation de la mélanine est particulièrement
complexe et fait intervenir schématiquement les principales étapes suivantes :

Tyrosine ---> Dopa ---> Dopaquinone ---> Dopachrome ---> Mélanine

30 La tyrosinase (monophénol dihydroxyl phénylalanine : oxygen oxydo-reductase
EC 1,14,18,1) est l'enzyme essentielle intervenant dans cette suite de

réactions. Elle catalyse notamment la réaction de transformation de la tyrosine en Dopa (dihydroxyphénylalanine) grâce à son activité hydroxylase et la réaction de transformation de la Dopa en dopaquinone grâce à son activité oxydase. Cette tyrosinase n'agit que lorsqu'elle est à l'état de maturation sous
5 l'action de certains facteurs biologiques.

Une substance est reconnue comme dépigmentante si elle agit directement sur la vitalité des mélanocytes épidermiques où se déroule la mélanogénèse et/ou si elle interfère avec une des étapes de la biosynthèse de la mélanine soit en
10 inhibant une des enzymes impliquées dans la mélanogénèse soit en s'intercalant comme analogue structural d'un des composés chimiques de la chaîne de synthèse de la mélanine, chaîne qui peut alors être bloquée et ainsi assurer la dépigmentation.

15 Les substances les plus utilisées en tant que dépigmentants sont plus particulièrement l'hydroquinone et ses dérivés, en particulier ses éthers tels que le monométhyléther et le monoéthyléther d'hydroquinone. Ces composés, bien qu'ils présentent une efficacité certaine, ne sont malheureusement pas exempts d'effets secondaires du fait de leur toxicité, ce qui peut rendre leur
20 emploi délicat, voire dangereux. Cette toxicité provient de ce qu'ils interviennent sur des mécanismes fondamentaux de la mélanogénèse en tuant des cellules qui risquent alors de perturber leur environnement biologique et qui par conséquent obligent la peau à les évacuer en produisant des toxines.

25 Ainsi, l'hydroquinone est un composé particulièrement irritant et cytotoxique pour le mélanocyte, dont le remplacement, total ou partiel a été envisagé par de nombreux auteurs.

On a ainsi cherché des substances qui n'interviennent pas dans le mécanisme
30 de la mélanogénèse mais qui agissent en amont sur la tyrosinase en empêchant son activation et sont de ce fait beaucoup moins toxiques. On

utilise couramment comme inhibiteur de l'activation de la tyrosinase l'acide kojique qui complexe le cuivre présent dans le site actif de cette enzyme. Malheureusement, ce composé peut provoquer des réactions d'allergie ("Contact allergy to kojic acid in skin care products", Nakagawa M. et al., in
5 Contact Dermatitis, Jan. 95, Vol 42 (1), pp.9-13). Ce composé est également instable en solution, ce qui complique quelque peu la fabrication de la composition.

L'utilisation de substances dépigmentantes topiques inoffensives présentant
10 une bonne efficacité est tout particulièrement recherchée en vue de traiter les hyperpigmentations régionales par hyperactivité mélanocytaire telles que les mélasmas idiopathiques, survenant lors de la grossesse ("masque de grossesse" ou chloasma) ou d'une contraception oestro-progestative, les hyperpigmentations localisées par hyperactivité et prolifération mélanocytaire
15 bénigne, telles que les taches pigmentaires séniles dites lentigo actiniques, les hyperpigmentations ou dépigmentations accidentelles, éventuellement dues à la photosensibilisation ou à la cicatrisation post-lésionnelle, ainsi que certaines leucodermies, telles que le vitiligo. Pour ces dernières (les cicatrises pouvant aboutir à une cicatrice donnant à la peau un aspect plus blanc et les
20 leucodermies), à défaut de pouvoir repigmenter la peau lésée, on achève de dépigmenter les zones de peau normale résiduelle pour donner à l'ensemble de la peau une teinte blanche homogène.

Aussi, il subsiste le besoin d'un nouvel agent blanchissant de la peau humaine,
25 des poils et/ou des cheveux à action aussi efficace que ceux connus, mais n'ayant pas leurs inconvénients, c'est-à-dire qui soit non irritant, non toxique et/ou non allergisant pour la peau et stable dans une composition.

La demanderesse a trouvé de manière inattendue que le paracétamol
30 présentait la propriété d'inhiber la tyrosinase, et donc la synthèse de la mélanine, et pouvait ainsi agir efficacement sur la pigmentation et les taches de

la peau ou sur la pigmentation des poils et/ou des cheveux sans une quelconque toxicité.

Le paracétamol est le N-(4-hydroxyphenyl)acétamide (ou acétaminophène).

5

Il est connu depuis de nombreuses années comme agent antipyrétique et analgésique. Toutefois personne jusqu'à ce jour n'avait envisagé de l'utiliser comme agent dépigmentant.

10 La présente invention a donc pour objet l'utilisation du paracétamol dans et/ou pour la fabrication d'une composition cosmétique et/ou dermatologique pour dépigmenter et/ou blanchir la peau humaine et/ou enlever les taches pigmentaires de la peau et/ou dépigmenter les poils et/ou les cheveux.

15 La présente invention a aussi pour objet l'utilisation du paracétamol dans et/ou pour la fabrication d'une composition cosmétique et/ou dermatologique, comme inhibiteur de la tyrosinase et/ou de la synthèse de la mélanine.

En effet, sans vouloir se lier à une quelconque théorie de l'invention, il semble
20 que le paracétamol agit non pas comme substrat de la tyrosinase, mais comme un inhibiteur des activités hydroxylase et oxydase de la tyrosinase.

La présente invention a aussi pour objet l'utilisation du paracétamol dans une composition cosmétique dépigmentante et/ou blanchissante de la peau
25 humaine, des poils ou des cheveux.

La présente invention a aussi pour objet une composition dépigmentante ou blanchissante, caractérisée en ce qu'elle contient, dans un milieu cosmétiquement et/ou dermatologiquement acceptable, du paracétamol. Cette
30 composition est plus particulièrement destinée à un usage topique sur la peau et/ou ses phanères (cheveux, poils et ongles).

La présente invention se rapporte également à un procédé cosmétique et/ou dermatologique de dépigmentation et/ou de blanchiment de la peau humaine, des poils ou des cheveux consistant à appliquer sur la peau, les poils ou les
5 cheveux une composition selon l'invention.

La composition selon l'invention est appropriée pour une utilisation topique et contient donc un milieu cosmétiquement ou dermatologiquement acceptable, c'est-à-dire compatible avec la peau, les poils ou les cheveux.

10

Le paracétamol peut être notamment présent en une quantité allant de 0,01 à 10 % et de préférence de 0,3 à 3 % du poids total de la composition.

La composition de l'invention peut se présenter sous toutes les formes
15 galéniques normalement utilisées pour une application topique, notamment sous forme d'une solution aqueuse, hydroalcoolique ou huileuse, d'une émulsion huile-dans-eau ou eau-dans-huile ou multiple, d'un gel aqueux ou huileux, d'un produit anhydre liquide, pâteux ou solide, d'une dispersion d'huile dans une phase aqueuse à l'aide de sphérules, ces sphérules pouvant être des
20 nanoparticules polymériques telles que les nanosphères et les nanocapsules ou mieux des vésicules lipidiques de type ionique et/ou non-ionique.

Cette composition peut être plus ou moins fluide et avoir l'aspect d'une crème blanche ou colorée, d'une pommade, d'un lait, d'une lotion, d'un sérum, d'une
25 pâte, d'une mousse. Elle peut éventuellement être appliquée sur la peau ou sur les cheveux sous forme d'aérosol. Elle peut également se présenter sous forme solide, et par exemple sous forme de stick. Elle peut être utilisée comme produit de soin et/ou comme produit de maquillage. Elle peut également être sous une forme de shampooings ou après-shampooings. Elle peut enfin se
30 présenter sous forme de patch.

De façon connue, la composition de l'invention peut contenir également les adjuvants habituels dans les domaines cosmétique et dermatologique, tels que les gélifiants hydrophiles ou lipophiles, les actifs hydrophiles ou lipophiles, les conservateurs, les antioxydants, les solvants, les parfums, les charges, les
5 filtres, les pigments, les absorbeurs d'odeur et les matières colorantes. Les quantités de ces différents adjuvants sont celles classiquement utilisées dans les domaines considérés, et par exemple de 0,01 à 20 % du poids total de la composition. Ces adjuvants, selon leur nature, peuvent être introduits dans la phase grasse, dans la phase aqueuse, dans les vésicules lipidiques et/ou dans
10 les nanoparticules.

Lorsque la composition de l'invention est une émulsion, la proportion de la phase grasse peut aller de 5 à 80 % en poids, et de préférence de 5 à 50 % en poids par rapport au poids total de la composition. Les huiles, les
15 émulsionnants et les coémulsionnants utilisés dans la composition sous forme d'émulsion sont choisis parmi ceux classiquement utilisés dans le domaine considéré. L'émulsionnant et le coémulsionnant sont présents, dans la composition, en une proportion allant de 0,3 à 30 % en poids, et de préférence de 0,5 à 20 % en poids par rapport au poids total de la composition.

20

Comme huiles utilisables dans l'invention, on peut citer les huiles minérales (huile de vaseline), les huiles d'origine végétale (huile d'avocat, huile de soja), les huiles d'origine animale (lanoline), les huiles de synthèse (perhydrosqualène), les huiles siliconées (cyclométhicone) et les huiles fluorées
25 (perfluoropolyéthers). On peut aussi utiliser comme matières grasses des alcools gras (alcool cétylique), des acides gras, des cires (cire de carnauba, ozokérite).

Comme émulsionnants et coémulsionnants utilisables dans l'invention, on peut
30 citer par exemple les esters d'acide gras et de polyéthylène glycol tels que le

stéarate de PEG-20, et les esters d'acide gras et de glycérine tels que le stéarate de glycéryle.

Comme gélifiants hydrophiles, on peut citer en particulier les polymères
5 carboxyvinyliques (carbomer), les copolymères acryliques tels que les
copolymères d'acrylates/alkylacrylates, les polyacrylamides, les
polysaccharides, les gommes naturelles et les argiles, et, comme gélifiants
lipophiles, on peut citer les argiles modifiées comme les bentones, les sels
métalliques d'acides gras, la silice hydrophobe et les polyéthylènes.

10

Comme actifs, on peut utiliser notamment les polyols (glycérine, propylène
glycol), les vitamines, les agents kératolytiques et/ou desquamants (acide
salicylique et ses dérivés, alpha-hydroxyacides, acide ascorbique et ses
dérivés), les agents anti-inflammatoires, les agents apaisants et leurs
15 mélanges. On peut également associer le paracétamol à d'autres agents
dépigmentants, tels que l'acide kojique ou l'hydroquinone et ses dérivés, ce qui
permet d'utiliser ces derniers à des doses moins toxiques pour la peau. En cas
d'incompatibilité, ces actifs et/ou le paracétamol peuvent être incorporés dans
des sphérules, notamment des vésicules ioniques ou non-ioniques et/ou des
20 nanoparticules (nanocapsules et/ou nanosphères), de manière à les isoler les
uns des autres dans la composition.

La composition est généralement appliquée sur la partie (peau ou muqueuses)
à traiter, puis peut être éventuellement maintenue en position par une pièce
25 (patch) adhésive. La pièce adhésive peut permettre une occlusion partielle ou
totale de la composition sur la partie à traiter. On peut par exemple utiliser,
comme pansement adhésif, le produit Tegaderm® vendu par les Laboratoires
3M Santé ou, comme pièce adhésive, le produit Finn Chambers® vendu par la
société Promédica.

30

Tests :

- Des tests ont mis en évidence l'activité du paracétamol comme inhibiteur de la tyrosinase par évaluation de ses effets inhibiteurs sur l'activité tyrosine hydroxylase et sur l'activité dopa-oxydase de la tyrosinase de champignons.

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Selon ce test, on suit par spectrométrie visible à 475 nm la quantité de dopachrome formée (produit intermédiaire avant les réactions d'oxydation non enzymatiques aboutissant à la formation de mélanine) en fonction du temps. Ces réactions sont catalysées in vitro par la tyrosinase de champignons (5µg à
10 3400 unités/mg, vendue par Sigma) en présence d'une part de 250µM de L-tyrosine (figure 1) et d'autre part de 250µM de L-dopa (figure 2) avec 100µM de paracétamol.

Le milieu réactionnel utilisé pour réaliser ce test correspond à 1 ml d'une
15 solution de tampon phosphate (Dulbecco's PBS) à pH = 7,4. Pour le témoin, la quantité d'inhibiteur a été remplacée par le même tampon phosphate à pH = 7,4. Le zéro des courbes a été également étalonné à l'aide du tampon phosphate.

20 Les courbes présentées sur les figures 1 et 2 ont été tracées en suivant, en ordonnée, la densité optique obtenue par la dopachrome formée en fonction du temps indiqué en abscisse et exprimé en secondes. Les courbes (a) (ou représentés par •) sont relatives au témoin et les courbes (b) (ou représentés par x) sont relatives au paracétamol.

25

En comparant les deux courbes d'une même figure, on voit nettement que la présence de paracétamol entraîne un net ralentissement de la quantité de dopachrome formée au cours du temps, ce qui signifie que le paracétamol présente un effet d'inhibition de la tyrosinase.

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- Par ailleurs, il a été réalisé le spectre d'absorption entre 190nm et 540nm pour suivre une éventuelle réaction du paracétamol avec la tyrosinase. Dans les mêmes conditions que précédemment avec des concentrations en paracétamol qui sont ici de 1mM et 100µM et avec un temps d'incubation de 2 heures à 5 30°C, on n'observe aucune modification de la densité optique par rapport au témoin. Ce qui permet de dire que le paracétamol est un très mauvais substrat de la tyrosinase.

- L'invention va maintenant être illustrée à l'aide des exemples qui suivent. Les 10 concentrations sont données en pourcentage en poids.

Exemple 1 : Crème traitante

- | | | |
|----|---|-----------|
| | - Alcool cétylique | 1,05 % |
| 15 | - Stéarate de PEG-20 (Myrj 49 vendu par la société ICI) | 2 % |
| | - Cyclométhicone | 6 % |
| | - Paracétamol | 0,5 % |
| | - Carbomer | 0,6 % |
| | - Glycérine | 3 % |
| 20 | - Triéthanolamine | 1 % |
| | - Conservateurs | 0,5 % |
| | - Eau déminéralisée | qsp 100 % |

- La crème obtenue utilisée en application quotidienne, permet d'obtenir un 25 blanchiment de la peau.

Exemple 2 : Gel traitant

- | | | |
|----|--------------------|------|
| 30 | - Propylène glycol | 10 % |
| | - Alcool éthylique | 40 % |

	- Glycérine	3	%
	- Paracétamol	0,5	%
	- Conservateurs	0,15	%
	- Parfum	0,15	%
5	- Eau déminéralisée	qsp 100	%

Le gel obtenu peut être utilisé quotidiennement et est apte à dépigmenter la peau.

10

Exemple 3 : Stick traitant

	- Cire de Carnauba	5	%
	- Ozokerite	7	%
15	- Lanoline	6	%
	- Dioxyde de titane (pigments)	20	%
	- Amidon de riz (charge)	7	%
	- EDTA	0,1	%
	- Paracétamol	2	%
20	- Perhydrosqualène	qsp 100	%

Le stick obtenu, utilisé sur les taches pigmentaires, permet de les atténuer voire de les faire disparaître.

25 Exemple 4 : Patch dépigmentant :

Le patch auto-adhésif contient 1% de paracétamol immobilisé.

Le patch est appliqué quotidiennement le soir sur une zone à dépigmenter comme les taches pigmentaires.

30

Exemple 4 :

Mise en évidence de l'effet dépigmentant par voie topique sur la peau d'une composition comprenant du paracétamol

5 Composition :

Paracétamol à 5% en poids par volume d'une solution constituée par : eau (22g), propylène glycol (23g) et alcool (55g).

Le traitement consiste à appliquer sur deux surfaces d'avant-bras de 1cm²
10 préalablement rasées 50µl de la composition d'une part (zone "paracétamol") et 50µl du véhicule (solution mentionnée ci-dessus sans le paracétamol) (zone "véhicule") d'autre part, et ceci matin et soir pendant plus d'un mois (du 17 octobre au 23 novembre). Cette application se fait à l'aide d'une pipette, on attend 10 secondes de contact avant de masser légèrement jusqu'à séchage
15 de la surface.

Le 19 novembre, on irradie les surfaces de peau préalablement traitées, soit par la composition, soit par le véhicule, avec des rayonnements UV-B pendant 6 minutes (environ 1 M.E.D.). On irradie également deux surfaces de la peau
20 rasée non traitée adjacentes aux deux autres zones qui servent de témoin (zone "témoin").

On suit la colorimétrie (colorimètre Minolta CR200®) pendant 4 jours de ces zones (le jour 0 correspondant au 19 novembre).

25 Trois domaines de colorimétrie sont pris en compte. Ainsi, de manière schématique, L, qui est la luminance, correspond à la clarté de la peau (plus L est grand, plus la peau est claire), A correspond à la rougeur de la peau (due notamment à l'érythème post-UV-B), B correspond à la couleur jaune de la peau (plus B est grand, plus il y a pigmentation mélanique).

La figure 3 représente la différence de luminance de la zone "paracétamol" (c) d'une part, de la zone "véhicule" (d) d'autre part avec la zone "témoin" correspondante en fonction du temps (exprimé en jour : nov correspond à novembre).

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La figure 4 représente la différence de rougeur de la zone "paracétamol" (c) d'une part, de la zone "véhicule" (d) d'autre part avec la zone "témoin" correspondante en fonction du temps (exprimé en jour).

- 10 La figure 5 représente la différence de jaune de la zone "paracétamol" (c) d'une part, de la zone "véhicule" (d) d'autre part avec la zone "témoin" correspondante en fonction du temps (exprimé en jour).

- On s'aperçoit que, dans ces trois domaines de colorimétrie, la zone
- 15 "paracétamol" correspond bien à une peau dépigmentée par rapport à la zone "véhicule".

REVENDICATIONS

1. Utilisation du paracétamol dans et/ou pour la fabrication d'une composition cosmétique et/ou dermatologique pour dépigmenter et/ou blanchir la peau humaine et/ou enlever les taches pigmentaires de la peau et/ou dépigmenter les poils et/ou les cheveux.
5
2. Utilisation du paracétamol dans et/ou pour la fabrication d'une composition cosmétique et/ou dermatologique, comme inhibiteur de la tyrosinase et/ou de la synthèse de la mélanine.
10
3. Utilisation du paracétamol dans une composition cosmétique dépigmentante et/ou blanchissante de la peau humaine, des poils ou des cheveux.
15
4. Composition dépigmentante ou blanchissante, caractérisée en ce qu'elle comprend, dans un milieu cosmétiquement et/ou dermatologiquement acceptable, du paracétamol.
- 20 5. Composition selon la revendication précédente, caractérisée en ce qu'elle est destinée à un usage topique sur la peau et/ou ses phanères.
6. Utilisation et composition selon l'une quelconque des revendications précédentes, caractérisée en ce que le paracétamol est présent en une quantité allant de 0,01 à 10 % du poids total de la composition.
25
7. Utilisation et composition selon la revendication précédente, caractérisée en ce que le paracétamol est présent en une quantité allant de 0,3 à 3 % du poids total de la composition.

8. Utilisation et composition selon l'une quelconque des revendications précédentes, caractérisée en ce que la composition comprend en outre, au moins un actif choisi parmi les agents kératolytiques et/ou desquamants, anti-inflammatoires, apaisants, autres agents dépigmentants et leurs mélanges.

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9. Utilisation et composition selon l'une des revendications précédentes, caractérisée en ce que le paracétamol et/ou l'actif est encapsulé dans des sphérules.

- 10 10. Procédé cosmétique de dépigmentation et/ou blanchiment de la peau humaine, des poils ou des cheveux, caractérisé en ce qu'il consiste à appliquer sur la peau, les poils ou les cheveux une composition décrite selon l'une des revendications précédentes 4 à 9.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/FR 97/02084

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K7/48 A61K7/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 24179 A (PROCTER & GAMBLE) 14 September 1995 see page 11, line 21; claims 1-10 -----	1-10

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 9524179 A	14-09-95	AU 1982595 A	25-09-95
		CA 2185231 A	14-09-95
		CN 1145582 A	19-03-97
		CZ 9602673 A	12-03-97
		EP 0748203 A	18-12-96
		JP 9510208 T	14-10-97

RAPPORT DE RECHERCHE INTERNATIONALE

De l'Etat de l'Union internationale No

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A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 6 A61K7/48 A61K7/06

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)

CIB 6 A61K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	WO 95 24179 A (PROCTER & GAMBLE) 14 septembre 1995 voir page 11, ligne 21; revendications 1-10 -----	1-10



Voir la suite du cadre C pour la fin de la liste des documents



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Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
WO 9524179 A	14-09-95	AU 1982595 A	25-09-95
		CA 2185231 A	14-09-95
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(74) Agent: ERNEST GUTMANN-YVES PLASSERAUD S.A.; 3, rue Chauveau-Lagarde, F-75008 Paris (FR).			
(54) Title: RESISTANCE AGAINST NEMATODES AND/OR APHIDS			
(57) Abstract			
<p>The invention relates to genes capable of conferring resistance against nematodes and/or aphids. Preferred nucleic acids of the invention are DNA sequences which are at least part of the DNA sequence provided on figures or homologous thereto. The invention further relates to vectors, cells and seeds comprising said nucleic acids, as well as genetically transformed plants which are resistant to nematodes and/or aphids. The invention also relates to oligonucleotides, primers, diagnostic kit and polypeptides.</p>			

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RESISTANCE AGAINST NEMATODES AND/OR APHIDS

FIELD OF THE INVENTION

The present invention relates to resistance genes, DNA constructs, micro-organisms, plant cells and plants comprising said resistance genes. Furthermore the invention relates to genetically transformed plants which are resistant against nematodes and/or aphids. In addition, the invention relates to probes, and primers for the identification of the resistance genes and diagnostic kits comprising said probes and/or primers. Finally, the invention relates to polypeptides encoded by said resistance genes and the use of said polypeptides.

BACKGROUND OF THE INVENTION

Plant pathogens are responsible for substantially losses of plants and plant products due to infection of the plant. Plant diseases, as a result of infection by plant pathogens or pests, cause damage to the plants and/or plant products, reduce production and yield, limit the kind of plants that can grow in certain geographic areas and as a result cause severe (financial) losses to the grower.

Plant parasitic nematodes occur worldwide and most of them live most of their life in the topsoil layer. Although losses caused by direct feeding of nematodes on plant roots is considered to be of minor importance, several species, among them the root-knot nematodes belonging to the *Meloidogyne* species, the cyst nematodes belonging to the *Heterodera* species and *Globodera* species and other nematodes such as the *Nacobbus* species, cause severe damage and economic crop losses. Root-knot nematodes also occur throughout the world but are found more frequently and in greater numbers in areas with warmer climates and in greenhouses. The most important *Meloidogyne* species are *M. incognita*, *M. arenaria*, *M. hapla* and *M. javanica*, of which *M. hapla* also occurs in more temperate climatic zones.

Different means for control of the plant pathogens exist, such as mechanical cultivation of the soil, chemical treatment with pesticides, including nematicides and insecticides, or crop rotation. However, for certain plant pathogens, especially nematodes, these means of control are insufficient to protect the plants from

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infection and resulting diseases. The only effective means of control involves plant host resistance (Russell, 1978, Plant Breeding for pest and disease resistance, Butterworths edit., 485 pp). The development of cultivars resistant to common plant pathogens is one of the major goals of plant breeders today, in order to reduce or ultimately eliminate the extensive need for pesticides. The burden for the environment of the large amounts of pesticides injected into the soil or sprayed on crops, trees etc. worldwide each year becomes too severe. Moreover, governmental regulations in Western countries restrict the use or even forbid the use of certain pesticides. Therefore, the need for plants which are resistant to one or more of their pathogens, or which have a reduced susceptibility to their attackers becomes more and more pressing. The development of resistant plants is one of the important objectives of current plant breeding programs. Plant genotypes susceptible for particular pathogens are crossed with resistant plant genotypes in order to introduce the resistant phenotype into the breeding line.

Damage by root-knot nematodes results primarily from the invasion of the plant roots by larvae which in a compatible relationship with the plant develop into a reproducing female. After invasion the larvae cause root cells to develop into giant cells on which they feed. Upon infection galls or knots are formed on the roots and the plant roots become otherwise disturbed, thickened and stunted. The root system thus disfunctions in the uptake of water and nutritional elements which damages the plant growth and development. Frequently damage to infected plants is increased by parasitic fungi attacking the weakened root tissue. Infected plants show reduced growth and smaller pale coloured leaves, with dwarf poor quality fruits or even without fruits, and tend to wilt in warmer climates (Agrios, 1988 in: Plant Pathology, Academic Press, Inc.). The damage and/or yield reduction caused by root-knot nematodes is substantial on the total agricultural production worldwide. In individual stand yield losses can be as high as 25-50 %, or even a crop may be killed.

In greenhouses root-knot nematodes can be controlled with steam sterilization of the soil or soil fumigation with nematicides. Under field conditions control can be achieved by the use of nematicides. However, the use of such, in

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some cases very persistent, chemicals is increasingly debated and in some countries the use of certain nematicides is even forbidden.

Breeding genetically resistant genotypes is the most reliable and effective way of controlling root-knot disease. For a number of crop species the availability of resistance within the related germplasm has been reported, e.g. potato, cotton, tobacco, wheat, soybean, tomato, eggplant, common bean and alfalfa. Resistance breeding is hampered by firstly the limited occurrence of (known) resistance genes in the available germplasm, secondly, in some plant species the existence of crossing barriers between the cultivated crop species and the resistance bearing related species, and thirdly, screening tests for resistance versus susceptibility to nematodes are laborious and often not reliable. Therefore, resistance breeding is very difficult or not to achieve, or if possible time consuming.

Successful introduction of resistance genes has been realized in tomato. The resistance gene *Mi* (*Meloidogyne incognita*) has been introduced into cultivated tomato, *Lycopersicon esculentum*, after crossing with the related wild species *L. peruvianum* (PI 128657), using embryo culture. The *Mi* gene confers resistance to various *Meloidogyne* spp. (Fassuliotis, 1991, in: Genetic Improvement of Tomato, Springer Verlag edit.). The *Mi* resistance gene is reported to be a monogenic dominant gene (Gilbert and McGuire, 1956, Proc. Am. Soc. Hortic. Sci. 68, 437-442) and is located on tomato chromosome 6. It is also postulated that the introgressed region comprising the *Mi* locus is involved in conferring resistance to potato aphid (*Macrosiphum euphorbia*) (Kaloshian et al, 1995, Proc. Natl. Acad. Sci. USA, 92, 622-625).

Plants have developed a complex defense mechanism against attack and infection by pathogens. To date, the exact mechanism of their defense system is not yet elucidated.

Nematode resistance in tomato is expressed after penetration. After the juvenile larva enters the root and establishes itself at a feeding site, a hypersensitive reaction (HR) adjacent to the head of the nematode is triggered that results in local death of the host cells. The nematode is also adversely affected by this HR and dies (Fassuliotis, 1991, in: Genetic Improvement of Tomato, Springer Verlag edit.).

Whether or not there exists a gene-for-gene relationship sensu Flor (1956, Adv. Gen. 8, 29-54) as is frequently the case in other plant-pathogen relationships where resistance is based on HR-incompatibility is unknown.

The isolation of plant genes without knowing their gene products is very laborious and difficult, because of the enormous genome sizes of plant species: e.g. tomato has a genome size of 1000 Mb (10^9 base pairs of nuclear DNA), maize has a genome size of 3000 Mb and wheat has even more than 16×10^9 base pairs. Searching for a specific gene among these billions of base pairs is only feasible when (i) there are enough molecular markers tightly linked to the gene of interest and (ii) there is good genetic material available (Tanksley *et al.*, 1995, Trends in Genetics, 11, p. 63-68).

Although, the isolation of a few resistance genes has been reported, none of these resistance genes are able to confer the host plant resistant to nematodes or to aphids. Examples of such isolated resistance genes are: *RPS2* from Arabidopsis (resistance to *Pseudomonas syringae* expressing *avrRpt2*), *N* from tobacco (resistance to tobacco mosaic virus), *Cf-9* from tomato (resistance to the leaf fungal pathogen *Cladosporium fulvum* carrying *avr9*) and *L⁶* from flax (resistance to the corresponding leaf rust fungal race) (Dangl, 1995, Cell 80, 363-366).

The present invention provides the first isolated nematode resistance gene, and furthermore, provides the first isolated aphid resistance gene. Moreover, the present invention relates to a dual function resistance gene conferring reduced susceptibility to nematodes as well as aphids, and preferably to *Meloidogyne incognita* and *Macrosiphum euphorbiae* respectively.

SUMMARY OF THE INVENTION

The present invention relates to a nucleic acid comprising the *Mi* resistance gene which when present and expressed in a plant is capable of conferring to said plant resistance against nematodes and/or aphids. Furthermore, the invention relates to the *Mi* resistance gene of which the DNA sequence is disclosed herein. The invention also relates to a gene product encoded by the *Mi* resistance gene. In addition, the present invention relates to DNA constructs, cosmids, vectors, bacterial

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strains, yeast cells and plant cells comprising the *Mi* resistance gene. In another aspect, the present invention relates to a genetically transformed plant, which is resistant to a nematode, said nematode being capable of infecting the untransformed plant. Furthermore, the invention relates to resistance genes which
5 are homologous to the *Mi* resistance gene, and which, when present in a plant, are able of conferring said plant resistance to infection by pathogens.

Moreover, the present invention relates to a nucleic acid comprising the *Meu-1* resistance gene which when present in a plant is capable of conferring to said plant reduced susceptibility to aphids. In particular the *Meu-1* resistance
10 gene corresponds to the *Mi* resistance gene. Especially the *Meu-1* resistance gene has the same nucleotide sequence as the *Mi* resistance gene. Thus, the present invention also relates to genetically transformed plants, which are reduced susceptible, and preferably resistant to aphids, in particular to potato aphids.

15 Finally, the invention relates to oligonucleotides corresponding to the sequence of the said resistance gene or part thereof, and detection kits comprising said oligonucleotides.

DESCRIPTION OF THE FIGURES

20 Figure 1 shows a physical map of YAC 1/1172, YAC 2/1256 and YAC 1/1084, with a size of 570, 500 and 470 kb respectively. The position of the *Sfi*I and *Bss*HI restriction sites and the size of the restriction fragments are indicated. The location of the various AFLP markers on the restriction fragments are indicated.

Figure 2 shows a schematic drawing of the binary cosmid vector pJJ04541
25 which is used to construct a cosmid library of YAC 1/546. Plasmid pRK290 (20 kb large) (Ditta *et al*, 1980, Proc. Natl. Acad. Sci. USA, 77, 7347-7351) was used as starting vector. "Tet" refers to the gene conferring resistance to tetracyclin. "LB" signifies T-DNA left border repeat sequence, and "RB" signifies the right border repeat. The cauliflower mosaic virus 35S promoter sequence is indicated by "p35S",
30 and "ocs3" indicates the octopine synthase 3' end. "NPT" indicates neomycin phosphotransferase, and "cos" refers to the bacteriophage lambda cos site enabling

in vitro packaging. "pDBS" indicates the polylinker of pBluescript (Stratagene, La Jolla, CA, USA).

Figure 3A shows a schematic representation of the detailed position of the AFLP markers on YAC 1/1172, YAC 2/1256 and YAC 1/1084. Positioning is based on the cosmid contig constructed for the various defined regions.

Figure 3B shows a schematic representation of the cosmid contig of the region comprising the *Mi* resistance gene. The cosmids Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14 are represented by horizontal lines. The location of the AFLP markers PM14 and PM25 is indicated.

Figure 4 shows a physical fine map of the cosmids Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14 for the restriction enzyme *Pst*I. The size of the *Pst*I fragments is indicated (in kb). The *Mi* phenotype, as identified in an *in vitro* disease assay, of the R_0 plants comprising the various cosmids is indicated in the right end part of the figure. The DNA segment of which the nucleotide sequence was determined is indicated by a double line with a bidirectional arrow.

Figure 5 shows the nucleotide sequence of a DNA segment of approximately 9.9 kb around the AFLP marker PM14, and a deduced amino acid sequence of the *Mi* resistance gene. The initiation codon (ATG position 3263-3265) is underlined and the termination codon (TAG position 7109-7111) is double underlined, showing an open reading frame (ORF1) encoding a polypeptide of 1257 amino acids (fig. 7A). The *Mi* resistance gene comprises two intron sequences (shown in italics): one intron of 1306 nucleotides from position 1936 to position 3241 and one intron of 75 nucleotides from position 3305 to position 3379.

A second initiation codon (ATG position 3491-3493) which is in frame with the first initiation codon, results into a second open reading frame (ORF2) encoding a truncated polypeptide of 1206 amino acids (figure 7B).

The position of the AFLP marker PM14 is from nucleotide position 6921 (5'-TGCAGGA-3') to nucleotide position 7034 (5'-AGATTA-3').

Figure 6 shows a physical map of cosmids Mi-11 and Mi-18 and the determined nucleotide sequence of cosmid Mi-11. The sequence is divided in four contigs: con25 (5618 bp), con10 (898 kb), con62 (2495 bp) and Mi (9870 bp).

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The lower part of the figure depicts the presence ("+") or absence ("-") of several PCR fragments, corresponding to parts of the DNA segment of Figure 5, which are represented as horizontal lines of different lengths at the right hand side of the table, in the various genetic backgrounds (YAC clone 2/1256, *E. coli* containing cosmid Mi-11, *A. tumefaciens* containing cosmid Mi-11, *E. coli* containing cosmid Mi-18, *A. tumefaciens* containing cosmid Mi-18, resistant tomato line E22, susceptible tomato line 52201, R_0 plants transformed with cosmid Mi-11 and R_0 plants transformed with cosmid Mi-18).

Nucleotide sequence of cosmid Mi-11 and cosmid Mi-18. Analysis of different contigs.

Figure 7 A: shows the deduced amino acid sequence of the polypeptide encoded by ORF1.

B: shows the deduced amino acid sequence of the truncated polypeptide encoded by ORF2.

Figure 8 depicts a schematic representation of the structure of the Mi-resistance gene.

Figure 9 depicts a schematic representation of the Mi-resistance gene family.

DETAILED DESCRIPTION OF THE INVENTION

In the description and examples that follow, a number of terms are used herein. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

- nucleic acid: a double-stranded DNA molecule. The nucleic acid can be genomic DNA, cDNA, synthetic DNA or any other DNA;
- oligonucleotide: a short single-stranded DNA molecule;
- primers: in general, the term primer refers to a single-stranded DNA molecule which can prime the synthesis of DNA;
- nucleic acid hybridization: a method for detecting related DNA sequences by hybridization of single-stranded DNA on supports such as nylon membrane or nitrocellulose filter papers. Nucleic acid molecules that have complementary

base sequences will reform the double-stranded structure if mixed in solution under the proper conditions. The double-stranded structure will be formed between two complementary single-stranded nucleic acids even if one is immobilized on a support. In a Southern hybridization procedure, the latter situation occurs;

- 5 - hybridization probe: to detect a particular DNA sequence in the Southern hybridization procedure, a labelled DNA molecule or hybridization probe is reacted to the fractionated DNA bound to a support such as nylon membrane or nitrocellulose filter paper. The areas on the filter that carry DNA sequences complementary to the labelled DNA probe become labelled themselves as a
10 consequence of the reannealing reaction. The areas of the filter that exhibit such labelling can then be detected according to the type of label used. The hybridization probe is generally produced by molecular cloning of a specific DNA sequence or by synthesizing a synthetic oligonucleotide;
- 15 - homologous sequence: a sequence which has at least 50 %, preferably 60 %, more preferably 70 %, most preferably 80 % or even 90 % sequence identity with the particular sequence, whereby the length of sequences to be compared for nucleic acids is generally at least 120 nucleotides, preferably 200 nucleotides and more preferably 300 nucleotides and the length of
20 sequences to be compared for polypeptides is generally at least 40 amino acid residues, preferably 65 amino acid residues and more preferably 100 amino acid residues. Alternatively, a homologous sequence refers to a sequence which can hybridize under stringent conditions to a particular sequence, and/or a DNA sequence coding for a polypeptide which has
25 substantially the same properties as the polypeptide encoded by the particular DNA sequence, and/or a DNA sequence coding for a polypeptide having the same amino acid sequence as the polypeptide encoded by the particular DNA sequence and/or an amino acid sequence in which some amino acid residues have been changed with respect to the amino acid
30 sequence of the particular polypeptide without substantially affecting the major properties of said polypeptide;

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- stringent conditions refer to hybridization conditions which allow a nucleic acid sequence to hybridize to a particular sequence. In general, high stringent conditions refer to the hybridization conditions which allow a nucleic acid sequence of at least 50 nucleotides and preferably about 200 or more nucleotides to hybridize to a particular sequence at about 65 °C in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength, and washing at 65 °C in a solution comprising about 0,1 M salt, or less, preferably 0,2 x SSC or any other solution having a comparable ionic strength. These conditions allow the detection of sequences having about 90 % or more sequence identity. In general, lower stringent conditions refer to the hybridization conditions which allow a nucleic acid sequence of at least 50 nucleotides and preferably about 200 or more nucleotides to hybridize to a particular sequence at about 45 °C in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength, and washing at room temperature in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength. These conditions allow the detection of sequences having up to 50 % sequence identity. The person skilled in the art will be able to modify these hybridization conditions in order to identify sequences varying in identity between 50 % and 90 %;
- promoter: a transcription regulation region upstream from the coding sequence containing the regulatory sequences required for the transcription of the adjacent coding sequence and includes the 5' non-translated region or so called leader sequence of mRNA;
- terminator: a region downstream of the coding sequence which directs the termination of the transcription, also called the 3' non-translated region, which includes the poly-adenylation signal;
- resistance gene: a nucleic acid comprising a coding sequence as depicted in Figure 5, or part thereof, or any corresponding or homologous sequence;
- nematode(s): *Meloidogyne* spp. such as *Meloidogyne incognita*, *M. arenaria* or *M. javanica*, or any other genotype which is not able to infect a host having

a resistance gene according to the invention, such as but not limited to other root-knot nematodes, such as *M. hapla*, cyst nematodes such as *Heterodera* spp. or *Globodera* spp., or other nematodes such as *Nacobbus* spp., insects, such as potato aphid or any other plant pathogen or pest;

- 5 - resistance gene product: a polypeptide having an amino acid sequence as depicted in Figure 5, or part thereof, or any homologous amino acid sequence;
- R_0 plant: primary regenerant from a transformation experiment, also denoted as transformed plant or transgenic plant;
- 10 - R_1 line: the progeny of a selfed R_0 plant.
- R_2 line: the progeny of a selfed R_1 plant.
- 15 - R_1BC line: the progeny of a backcross between a R_1 plant and a plant of the genotype which was originally used for the transformation experiment.

In the present invention we have been able to identify and isolate the *Meloidogyne incognita* (*Mi*) resistance gene. The gene was cloned from a tomato
20 genotype which is resistant to *Meloidogyne incognita*. The isolated *Mi* resistance gene according to the invention can be transferred to a susceptible host plant using *Agrobacterium* mediated transformation or any other known transformation method, and is involved in conferring to the host plant resistance against plant pathogens, especially to nematodes. The host plant can be tomato or any other genotype that is
25 infected by said plant pathogen.

The present invention provides also a nucleic acid sequence comprising the *Mi* resistance gene, which is depicted in Figure 5.

With the *Mi* resistance gene according to the invention, one has an effective means of control against plant pathogens and/or pests, since the gene can be used
30 for transforming susceptible plant genotypes thereby producing genetically transformed plants having a reduced susceptibility or being preferably resistant to a plant pathogen or pest. In particular, a plant which is genetically transformed with the

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Mi resistance gene according to the invention has a reduced susceptibility to root-knot nematodes.

In a preferred embodiment the *Mi* resistance gene comprises the coding sequence provided in Figure 5 or any corresponding or homologous sequence or cDNA sequence, preceded by a promoter region and followed by a terminator region. The promoter region should be functional in plant cells and, preferably, corresponds to the native promoter region of the *Mi* resistance gene. However, it should be recognized that any heterologous promoter region can be used in conjunction with the coding sequences, as long as it is functional in plant cells. Preferably, a constitutive promoter is used, such as the CaMV 35 S promoter or T-DNA promoters, all well known to those skilled in the art. Furthermore, a suitable terminator region should be functional in plant cells all well known to those skilled in the art.

In addition the invention relates to the *Mi* resistance gene product which is encoded by the *Mi* resistance gene according to the invention and which has a deduced amino acid sequence provided in Figure 5 and Figure 7A, or which is homologous to the deduced amino acid sequence or part thereof. Furthermore, the *Mi* resistance gene product or a truncated polypeptide as provided in figure 7B can be used for raising antibodies against it, which antibodies can be used for the detection of the presence of the *Mi* resistance gene product.

In another aspect of the invention, the *Mi* resistance gene can be used for the design of oligonucleotides which are complementary to one strand of the DNA sequence as described in Figure 5, or part thereof, which can be used as hybridization probes, being accordingly labelled to allow detection, for the screening of genomic DNA or cDNA libraries for homologous genes. Homologous sequences which can hybridize to the probe under stringent hybridization conditions, and which encode for a gene product that is involved in conferring reduced susceptibility or resistance to a plant against a plant pathogen which normally infects said plant, are comprised within the scope of the present invention.

In another aspect of the invention oligonucleotides are designed based on the *Mi* resistance gene sequence, such that they can be used as hybridization probes in

Southern analysis. These probes can be used as molecular markers to distinguish plant genotypes having the resistance gene and plant genotypes lacking the resistance gene. Such a probe can be used as an additional tool in selection. In a preferred embodiment of the invention, oligonucleotides are designed based on the *Mi* resistance gene sequence, such that they can be used as primers in an amplification reaction, such as polymerase chain reaction (PCR), whereby the formation of an amplification product indicates the presence of the *Mi* resistance gene in a certain plant genotype. In a particular embodiment of the invention said primers direct the amplification of polymorphic fragments, so called molecular markers, which are closely linked to the *Mi* resistance gene. In a preferred embodiment said primers are used in selective restriction fragment amplification to identify AFLP markers, which are closely linked to the *Mi* resistance gene. The invention also relates to diagnostic kits, comprising oligonucleotides according to the invention, for the detection of the presence or absence of the *Mi* resistance gene within a genotype under study. Such a diagnostic kit circumvents the use of a laborious disease assay to screen for genotypes having the resistance gene or not.

Furthermore the invention relates to DNA constructs comprising a DNA sequence corresponding to the coding sequence of the *Mi* resistance gene and regulatory sequences functional in plant cells, said DNA sequence can be genomic DNA, cDNA, synthetic DNA or DNA of any other origin. Said regulatory sequences are either homologous or heterologous to the coding sequences of the *Mi* resistance gene. Preferably, said DNA construct comprises a nucleic acid whose sequence is provided in Figure 5, or part thereof.

The invention relates also to DNA constructs comprising the regulatory sequences, and more preferably the promoter region of the *Mi* resistance gene in conjunction with a structural gene sequence heterologous to said regulatory sequences.

The invention relates also to a DNA vector comprising a DNA construct according to the invention. Suitable vectors can be cloning vectors, transformation vectors, expression vectors, etc..., which are well known to those skilled in the art.

Furthermore, cells harbouring a vector comprising a DNA sequence corresponding to the sequence as described in Figure 5 or part thereof, or homologous thereto, are within the scope of the invention. Moreover, cells carrying a DNA construct according to the invention, are within the scope of this invention.

5 In one preferred embodiment of the invention, a genetically transformed plant is obtained by introducing the *Mi* resistance gene within the genome of said plant, being susceptible to nematodes, using standard transformation techniques, wherein said genetically transformed plant is resistant to nematodes.

10 In another embodiment of the invention, the *Mi* resistance gene can be transferred, using generally known transformation techniques, to a heterologous systems, such as but not limited to melon, tobacco, *Arabidopsis thaliana*, potato, sugarbeet, rapeseed, cucumber, pepper, eggplant. A heterologous system refers to a plant species which is different from the plant species from which the resistance gene was isolated.

15 In yet another embodiment of the invention, the *Mi* resistance gene corresponds to the *Macrosiphum euphorbiae* (*Meu-1*) resistance gene, and is involved in conferring to plants, transformed with the gene according to the invention, resistance to insects and in particular to aphids.

20 The DNA sequence comprising the *Mi* resistance gene as provided in the present invention has numerous applications of which some are described herein but which are not limiting the scope of the invention.

The present invention will be further described in detail in view of the isolation of the *Mi* resistance gene present in tomato lines which are resistant to root-knot nematodes. For the isolation of the *Mi* resistance gene we have used a map-based
25 cloning (positional cloning) strategy, comprising the following steps:

- (1) identification of molecular markers linked to the *Mi* resistance gene,
- (2) construction of a high molecular weight genomic YAC library,
- (3) physical mapping of the molecular markers on the YAC clones and YAC
30 contig building,

- (4) construction of a cosmid library of the YAC clones harbouring the linked molecular markers,
- (5) physical fine mapping and cosmid contig building,
- (6) genetic characterization of tomato mutants susceptible to root-knot nematodes,
- 5 (7) transformation of susceptible plants with the cosmids forming the contig,
- (8) complementation analysis.

For the identification of molecular markers, we have used the selective restriction fragment amplification technology, hereinafter also denoted as AFLP™ technology, which randomly amplifies a subset of DNA fragments out of a complex mixture of many DNA fragments and said amplified fragments generate fingerprints that can be analyzed. In general, total DNA of different genotypes of the same plant species are subjected to the AFLP technology and the different AFLP fingerprints obtained from the different genotypes are compared. Fragments that are present in one genotype and absent in another genotype are polymorphic fragments and are denoted as AFLP markers. The selectivity in AFLP reactions is obtained by using randomly chosen selective nucleotides at the 3' end of the PCR primers immediately adjacent to the nucleotides of the restriction enzyme site. In an AFLP screening the DNA to be studied is subjected to different primer combinations. The total amount of different primers that can be used is determined by the number of selective nucleotides that are added to the 3' end (4 primers with 1 selective nucleotides, 16 primers with 2 selective nucleotides, 64 primers with 3 selective nucleotides). If two different restriction enzymes are used than there are twice the amount of primers. Those primers can be used in different combination. If all possible combinations are used in an AFLP screening, than all the fragments present should have been amplified with one of the primer combinations (Zabeau and Vos, EP 0534858).

For the identification of AFLP markers linked to the *Mi* resistance gene different tomato lines were subjected to an AFLP screening. In a first step, two sets of nearly isogenic lines for nematode resistance versus susceptibility were analyzed by AFLP fingerprinting using the following primers:

*Pst*I-primers: 5'-GACTGCGTACATGCAGNN-3'

*Mse*I-primers: 5'-GATGAGTCCTGAGTAANN-3'

5 The N's indicate the variable selective nucleotides. In the AFLP screening all
16 primers possible for the *Pst*I-primer and all 64 primers possible for the *Mse*I-
primer were used on the two sets of nearly isogenic lines, giving a total of 16 x 64 =
1024 tested primer combinations. Upon analysis of all the AFLP fingerprints a total of
30 candidate AFLP markers linked to the *Mi* resistance gene were identified. These
10 candidate markers were subsequently tested on a panel of nematode resistant and
nematode susceptible tomato lines for confirmation and distance of linkage to the *Mi*
locus. The *Mi* resistance gene was introgressed in the cultivated tomato in 1944 from
Lycopersicon peruvianum. Modern nematode resistant tomato lines have been
subjected to numerous cycles of crossing expected to result in a small introgressed
15 region from *Lycopersicon peruvianum* with the *Mi* resistance gene. Testing of the
candidate AFLP markers on these modern tomato genotypes is expected to be a
good test for assessing close linkage to the *Mi* locus. A panel of 7 resistant and 11
susceptible tomato genotypes was tested with the candidate AFLP markers. A total
of 20 AFLP markers appeared to be present in all resistant lines and absent in all
20 susceptible lines and are referred to as *Mi* linked AFLP markers.

Next, four of the AFLP markers were screened on a high molecular weight
genomic library. The cloning of very large segments of DNA as large artificial
chromosomes in yeast has become an essential step in isolating genes via
positional cloning. The cloning capacity of the YAC vector allows the isolation of
25 DNA fragments up to one million base pairs in length. The tomato line *Lycopersicon*
esculentum E22, homozygous for the *Mi* locus, was used as source DNA to
construct a YAC library. We obtained a YAC library containing 3840 clones with an
average insert size of 520 Kb, representing approximately 2.2 genome equivalents
of the tomato genome. Three positive YAC clones were obtained after the AFLP
30 screening with the *Mi* linked AFLP markers: 1/1084, 1/1172 and 2/1256.
Subsequently, the presence of all *Mi* linked AFLP markers was determined in the 3

YAC clones. All markers appeared present in one or more of the 3 YAC clones, which allowed a first positioning of the various *Mi* linked AFLP markers. The AFLP data indicated that the 3 YAC clones constituted an overlapping contig of approximately 1.4 Mb (see Figure 1).

5 To determine the physical size of the *Mi* locus comprising the *Mi* linked AFLP markers and comprised in YAC clones 1/1084, 1/1172 and/or 2/1256 a long-range restriction map of the YAC contig was constructed. This defined a DNA segment comprising the *Mi* locus of about 700 kb on which all the *Mi* linked AFLP markers were located (see Figure 1).

10 A size of 700 kb is still too large for direct localization of the *Mi* resistance gene. Such large inserts cannot be transformed into plant cells directly. Therefore, a cosmid library was constructed of the yeast strain containing YAC 1/1172 and a cosmid library was constructed of the yeast strain containing YAC 2/1256 using cosmid vectors which are suitable for *Agrobacterium* mediated transformation. The
15 size of this binary cosmid vector amounts 29 kb and is shown schematically in Figure 2. The cloning capacity of this binary cosmid vector, using phage lambda packaging extract is within the range of 9 to 24 kb. Two banks of approximately 250,000 cosmid clones each were obtained from size fractionated yeast DNA. The cosmid banks were screened by colony hybridization using as probes labelled restriction fragments
20 of the YACs. Positive cosmids clones were identified and in addition, the cosmids were grouped into seven defined regions covering the *Mi* region.

In the following step the set of cosmids of the seven defined regions were fingerprinted using restriction fragment amplification to determine their relative order. A cosmid contig covering a DNA segment of approximately 700 kb could be
25 constructed. Subsequently, the presence of the *Mi* linked AFLP markers in this cosmid contig was determined. A physical map of the DNA segment comprising the *Mi* resistance gene with the positions of the various *Mi* linked AFLP markers was obtained (see Figure 3).

A total of 96 overlapping cosmids together constituted the DNA segment
30 comprising the *Mi* resistance gene. Complementation analysis to identify the *Mi* resistance gene with such a large set of cosmids is a very laborious task. Therefore,

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the position of the *Mi* resistance gene on the cosmid contig was determined using mutant tomato lines. These mutant lines are members from a family originating from a common ancestor and contained a wild-type (nematode resistant) *Mi* genotype but a mutant nematode susceptible phenotype. Upon analysis with the set of *Mi* linked AFLP markers on a large number of these mutant lines three *Mi* linked AFLP markers appeared to be absent in most mutants. These AFLP markers, therefore showed a good correlation between the AFLP *Mi* genotype and the *Mi* phenotype, in contrast to all other 17 AFLP markers. Two of these AFLP markers, PM14 and PM25 were adjacent, and the region around these markers was assumed to be the most likely position for the *Mi* resistance gene. A set of 6 overlapping cosmids defining a DNA segment of approximately 50 kb around AFLP markers PM14 and PM25 was selected for complementation analysis (see Figure 4).

The final step in the identification of the *Mi* resistance gene via positional cloning is the complementation of the corresponding susceptible phenotype. The 6 cosmids from the candidate *Mi* region were introduced in *Agrobacterium tumefaciens* through conjugative transfer in a tri-parental mating. The presence of the cosmid in the *A. tumefaciens* strains was determined comparing various restriction enzyme patterns as well as DNA fingerprints from the *A. tumefaciens* strains with the *E. coli* strain containing the cosmid. Only those *A. tumefaciens* cultures harbouring a cosmid with the same DNA pattern as the corresponding *E. coli* culture were used to transform a susceptible tomato line. A susceptible tomato line was transformed with cosmids Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14 using standard transformation methods.

Roots of *in vitro* grown transformed R_0 plants were tested for disease symptoms in order to identify cosmids with the resistance gene. Root explants were transferred onto solidified medium in petri dishes and inoculated with ten galls from an axenic nematode culture of the root-knot nematode *Meloidogyne incognita*. Disease symptoms are scored six weeks after inoculation. A transgenic plant is considered resistant when no galls or one gall are visible on its root culture. A transgenic plant is considered susceptible when at least two galls have been induced on its root culture. The observations of the *in vitro* disease assay revealed

that 2 cosmids were able to complement the susceptible phenotype. The presence of the AFLP marker PM14 in the resistant R_0 plants indicated that the genomic insert present in cosmids Mi-11 and Mi-18 is also present in the R_0 plants and is involved in conferring the R_0 plants resistant to *Meloidogyne incognita*.

5 The primary regenerants (R_0 plants) of the transformation experiments were grown in the greenhouse for seed set to obtain R_1 lines which were tested for disease symptoms. The disease assay is performed on seedlings. Therefore, seeds are sown or small rooted plantlets are transferred into soil infected with *Meloidogyne incognita* and disease symptoms are scored 4 to 8 weeks after inoculation. Plants
10 are considered to be resistant when three or less galls are visible on the roots. Plants are considered to be susceptible when more than three galls are formed on the roots. The observations of the *in vivo* disease assay revealed that the resistant R_0 plants are corresponding to cosmid Mi-11 transformants.

 In order to confirm the stable integration of the *Mi* resistance gene into the
15 genome of the transgenic R_0 plants, resistant plants of the R_1 lines were selfed and grown in the greenhouse for seed set to obtain R_2 lines. Seedlings of the R_2 lines were subjected to an *in vivo* nematode disease assay. The results obtained indicated the stable inheritance of the *Mi* resistance gene.

 Finally, the inserts in cosmids Mi-11 and Mi-18 were further characterized.
20 Sequencing analysis revealed a large open reading frame (ORF2) of 3621 nucleotides. The DNA sequence is listed in Figure 5.

 The DNA sequence comprising the *Mi* resistance gene was further subjected to transcript mapping studies in order to determine the existence of intron sequences. These transcripts mapping studies were performed according to
25 generally known methods whereby genomic DNA sequences are compared with cDNA sequences. The comparison of cDNA sequences and genomic sequences revealed the existence of two intron sequences in the *Mi* resistance gene. One intron of 1306 nucleotides is located from nucleotide position 1936 to 3241 and a second intron of 75 nucleotides is located from nucleotide position 3305 to 3379, as is
30 depicted in Figure 5. The position of the transcription initiation site is postulated at or upstream of nucleotide 1880. The first ATG initiation codon is located at nucleotide

position 3263 which is 52 nucleotides upstream of the second intron, giving a large open reading frame (ORF1) encoding a polypeptide of 1257 amino acids (figure 7A).

Homology searches have shown that the polypeptides according to the invention belong to the LRR class of plant resistance proteins (Staskawicz et al, 1995, Science, 268, 661-667). In addition the protein can be divided into four regions designated A to D: region A comprises a high amount of leucine residues, region B comprises a nucleotide binding site motif, region C is the LRR region comprising 13 repeats with the following consensus sequence

a-a-NL-L-a-a-a/S- (Jones and Jones, 1997, Advances in Botanical Research, 24, 89-167) and region D reveals no homology to any known protein.

For the identification and isolation of homologous sequences falling within the scope of the present invention, genomic and cDNA libraries were screened with the coding sequence of the *Mi* resistance gene as a probe under stringent hybridization conditions. Positive clones were isolated and used for complementation analysis.

Southern blot hybridizations on the YAC contig have been performed with an internal *Pst*I fragment of the coding sequence of the *Mi* resistance gene. Three additional homologous regions could be identified: two in YAC 1/1172 and one in YAC 1/1084. Each region comprises 2 to 3 *Mi* homologues indicative of the fact that the *Mi* gene family is composed of about 10 to 12 members.

Surprisingly, aphid disease assays revealed that the R_0 plants, transformed with cosmid Mi-11, are resistant to *Meloidogyne incognita* as well as resistant to *Macrosiphum euphorbiae*, indicating that the genome insert present in cosmid Mi-11 is involved in conferring the R_0 plants resistant to nematodes as well as involved in conferring the R_0 plants resistant to aphids. In particular, a plant which is transformed with the resistance gene according to the invention has at least a reduced susceptibility to one or more pathogens, especially to root-knot nematodes and/or aphids.

In order to confirm the inheritance of the aphid resistance, (i) the previously obtained R_1 tomato lines which were derived from nematode resistant cosmid Mi-11 transformants, (ii) the R_2 lines derived from selfed nematode resistant R_1 plants and (iii) R_1 BC lines obtained from nematode resistant R_1

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plants backcrossed with susceptible tomato line 52201, were also tested for resistance against *M. euphorbiae*. The results obtained indicated the inheritance of the aphid resistance.

Cosmid Mi-11 was used for the transformation of nematode susceptible
5 genotypes of tobacco and potato, according to general known transformation methods. Roots of *in vitro* grown transformed R₀ plants of tobacco and potato were tested for disease symptoms as previously described herein. The observations of the disease assay on the root cultures of the transformed plants indicated that the cosmid is involved in conferring to the transformed plants a
10 reduced susceptibility to nematodes. The resistance gene according to the invention has an effect in reducing the susceptibility of a heterologous plant species to nematodes, preferably to *Meloidogyne spp.*, especially *Meloidogyne incognita*.

Furthermore tobacco transformants were also tested for aphid resistance,
15 and resistant R₀ plants could be identified.

The resistance gene according to the invention has a dual function and has an effect in heterologous systems.

Cosmid Mi-11 has been deposited on August 5, 1996 as plasmid pKGMi-11 at Centraalbureau voor Schimmelcultures at Baarn, The Netherlands, under deposit
20 number CBS 822.96.

Cosmid Mi-18 has been deposited on August 5, 1996 as plasmid pKGMi-18 at Centraalbureau voor Schimmelcultures at Baarn, The Netherlands, under deposit number CBS 821.96.

The following examples will provide a further illustration of the present
25 invention which is nevertheless not limited to these examples.

EXAMPLES**EXAMPLE 1: DISEASE ASSAY**

An axenic culture of the root-knot nematode *Meloidogyne incognita* is maintained on
5 sterile roots of the tomato cultivar MoneyMaker. The root cultures are grown on
solidified B5 medium (Gamborg *et al* 1968, Experimental Cell Research 50: 151-
158) with 2% saccharose and without hormones.

Root explants (1-5 cm), derived from *in vitro* grown transgenic tomato plants are
transferred onto the solidified B5 medium mentioned above to start root cultures. At
10 the same time each root explant is inoculated with ten galls from the axenic
nematode culture. The galls are placed a few centimetres from the root explant. The
Petri dishes with the roots and galls are incubated in the dark at 25°C. After four to
six weeks the level of infection is determined by counting the number of galls formed
on the root cultures.

15 The evaluation for resistance/susceptibility to *M. incognita* is as follows:

A transgenic plant is considered resistant when no or less than two galls are visible
on its root culture. A transgenic plant is considered susceptible when at least two
galls have been induced on its root culture.

20 **EXAMPLE 2: IDENTIFICATION OF AFLP MARKERS LINKED TO A DNA
SEGMENT COMPRISING THE *Mi* RESISTANCE GENE**

Tomato lines (*Lycopersicon esculentum*)

A total of 9 tomato lines resistant to *Meloidogyne incognita* and 13 tomato lines
25 susceptible to *M. incognita* were used to identify AFLP markers. Initially the AFLP
screening was performed on two sets of nearly isogenic lines 83M-R (resistant) and
83M-S (susceptible), and Motelle (resistant) and Mobox (susceptible). The candidate
markers resulting from this first screening were confirmed by a second screening on
7 *M. incognita* resistant and 11 *M. incognita* susceptible lines.

Two sets of nearly isogenic lines :

5	1.	83M-R	resistant	De Ruiter Zonen C.V., Bergschenhoek, The Netherlands (hereinafter "De Ruiter")
	2.	83M-S	susceptible	De Ruiter
	3.	Motelle	resistant	INRA, Montfavet, France
	4.	Mobox	susceptible	INRA, Montfavet, France
10	The 7 <i>M. incognita</i> resistant lines and 11 <i>M. incognita</i> susceptible lines for confirmation :			
15	5.	DR30	resistant	De Ruiter
	6.	DR17	resistant	De Ruiter
	7.	E22	resistant	Enza Zaden, de Enkhuizer Zaadhandel B.V., Enkhuizen, The Netherlands (hereinafter "Enza Zaden")
	8.	E1	resistant	Enza Zaden
20	9.	DR6	resistant	De Ruiter
	10.	DR10	resistant	De Ruiter
	11.	1872	resistant	Royal Sluis B.V., Enkhuizen, The Netherlands (hereinafter "Royal Sluis")
	12.	Moneymaker	susceptible	Agricultural University Wageningen
25	13.	DR12	susceptible	De Ruiter
	14.	DR23	susceptible	De Ruiter
	15.	GT	susceptible	De Ruiter
	16.	RZ3	susceptible	Rijk Zwaan Zaadteelt en Zaadhandel B.V., De Lier, The Netherlands (hereinafter "Rijk Zwaan")
30	17.	RZ5	susceptible	Rijk Zwaan
	18.	E3	susceptible	Enza Zaden
	19.	E7	susceptible	Enza Zaden

23

20.	E16	susceptible	Enza Zaden
21.	RS1	susceptible	Royal Sluis
22.	RS2	susceptible	Royal Sluis

5 Isolation and modification of the DNA

Total tomato DNA from the 22 lines described above was isolated from young leaves as described by Bernatzki and Tanksley (Theor. Appl. Genet. 72, 314-321). The typical yield was 50 - 100 µg DNA per gram of fresh leaf material. Template DNA for AFLP analysis with the enzyme combination *Pst*I-*Mse*I was prepared as described by Zabeau and Vos (European Patent Application, EP 0534858), and is described briefly below:

0.5 µg of tomato DNA was incubated for 1 hour at 37°C with 5 units *Pst*I and 5 units *Mse*I in 40 µl 10 mM Tris.HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA. Next 10 µl of a solution containing 5 pMol *Pst*I-adapters, 50 pMol *Mse*I-adapters, 1 unit T4 DNA-ligase, 1 mM ATP in 10 mM Tris.HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA was added, and the incubation was continued for 3 hours at 37°C. The adapters are depicted below:

20 The structure of the *Pst*I-adapter was:

5'-CTCGTAGACTGCGTACATGCA-3'

3'-CATCTGACGCATGT-5'

25 The structure of the *Mse*I-adapter was:

5'-GACGATGAGTCCTGAG-3'

3'-TACTCAGGACTCAT-5'

24

Adapters were prepared by adding equimolar amounts of both strands; adapters were not phosphorylated. After ligation, the reaction mixture was diluted to 500 μ l with 10 mM Tris.HCl, 0.1 mM EDTA pH 8.0, and stored at -20°C. The diluted reaction mixture is further referred to as template DNA.

5

AFLP reactions

The primers used for the AFLP screening are depicted below:

*Pst*I-primers: 5'-GACTGCGTACATGCAGNN-3'
10 *Mse*I-primers: 5'-GATGAGTCCTGAGTAANN-3'

The N's in the primers indicate that this part of the primers was variable. In the AFLP screening all 16 possible primers were used for the *Pst*I-primer and all 64 possible primers were used for the *Mse*I-primer. This gave a total of 16 x 64 combinations of
15 *Pst*I- and *Mse*I-primers, is 1024 primer combinations. All 1024 primer combinations were used in the AFLP screening for *Mi* linked AFLP markers. The AFLP reactions were performed in the following way:

AFLP reactions employed a radio-actively labelled *Pst*I-primer and a non-labelled
20 *Mse*I-primer. The *Pst*I-primers were end-labelled using (γ -³³P)ATP and T4 polynucleotide kinase. The labelling reactions were performed in 50 μ l 25 mM Tris.HCl pH 7.5, 10 mM MgCl₂, 5 mM DTT, 0.5 mM spermidine.3HCl using 500 ng oligonucleotide primer, 100 μ Ci (γ -³³P)ATP and 10 units T4 polynucleotide kinase. For AFLP analysis 20 μ l reaction mixture were prepared containing 5 ng labelled
25 *Pst*I-primer (0.5 μ l from the labelling reaction mixture), 30 ng *Mse*I-primer, 5 μ l template-DNA, 0.4 units Taq-polymerase, 10 mM Tris.HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM of all 4 dNTPs. AFLP reactions were performed using the following cycle profile: a 30 seconds DNA denaturation step at 94°C, a 30 seconds annealing step (see below), and a 1 minute extension step at 72°C. The annealing temperature
30 in the first cycle was 65°C, was subsequently reduced each cycle by 0.7°C for the next 12 cycles, and was continued at 56°C for the remaining 23 cycles. All

amplification reactions were performed in a PE-9600 thermocycler (Perkin Elmer Corp., Norwalk, CT, USA).

Gel analysis of AFLP reaction products

5 After amplification, reaction products were mixed with an equal volume (20 μ l) of formamide dye (98% formamide, 10 mM EDTA pH 8.0, and bromo phenol blue and xylene cyanol as tracking dyes). The resulting mixtures were heated for 3 minutes at 90°C, and then quickly cooled on ice. 2 μ l of each sample was loaded on a 5% denaturing (sequencing) polyacrylamide gel (Maxam and Gilbert, Methods in
10 Enzymology 65, 499-560). The gel matrix was prepared using 5% acrylamide, 0.25% methylene bisacryl, 7.5 M urea in 50 mM Tris/50 mM Boric acid/1 mM EDTA. To 100 ml of gel solution 500 μ l of 10% APS and 100 μ l TEMED was added and gels were cast using a SequiGen 38 x 50 cm gel apparatus (Biorad Laboratories Inc., Hercules, CA, USA). Sharktooth combs were used to give 97 lanes on the SequiGen
15 gel units. 100 mM Tris/100 mM Boric acid/2 mM EDTA was used as running buffer. Electrophoresis was performed at constant power, 110 Watts, for approximately 2 hours. After electrophoresis, gels were fixed for 30 minutes in 10% acetic acid dried on the glass plates and exposed to Fuji phospho image screens for 16 hours. Fingerprint patterns were visualized using a Fuji BAS-2000 phospho image analysis
20 system (Fuji Photo Film Company Ltd, Japan).

AFLP screening for linked markers

An AFLP screening was performed using all possible 1024 *Pst*I-*Mse*I primer combinations on the two sets of nearly isogenic lines. The aim was to identify AFLP
25 markers present in both resistant lines and absent in both susceptible lines. AFLP gels contained the AFLP fingerprints of 24 primer combinations of the 4 isogenic lines, giving a total of 43 gels. A total of 30 AFLP markers were identified present in both resistant lines and absent in both susceptible lines. These markers are referred to as candidate *Mi* linked AFLP markers.
30 Next, AFLP reactions were performed to determine the presence of the 30 candidate markers on the 7 resistant and 11 susceptible tomato lines. Of the 30 candidate

markers 20 markers appeared to be present in the 7 resistant lines and absent in the 11 susceptible lines. These 20 markers were used in further studies to map the *Mi* resistance gene. The primer combinations required to identify the 20 *Pst*I-*Mse*I markers are depicted in Table 1. In the column with the primer combinations, "*Pst*I-" refers to the sequence 5'-GACTGCGTACATGCAG-3' and "*Mse*I-" refers to the sequence 5'-GATGAGTCCTGAGTAA-3'. For example, marker PM14 can be identified using the *Pst*I-primer having the following sequence: 5'-GACTGCGTACATGCAGGA-3', and the *Mse*I-primer having the following sequence: 5'-GATGAGTCCTGAGTAATCT-3'.

10

TABLE 1

	marker	primer combination with selective extensions (NN/NNN)
15	PM02	<i>Pst</i> I-AT / <i>Mse</i> I-AAA
	PM07	<i>Pst</i> I-AA / <i>Mse</i> I-TAC
	PM08	<i>Pst</i> I-CT / <i>Mse</i> I-ACT
	PM10	<i>Pst</i> I-CA / <i>Mse</i> I-TCT
	PM11	<i>Pst</i> I-TA / <i>Mse</i> I-TGA
20	PM13	<i>Pst</i> I-GA / <i>Mse</i> I-ATC
	PM14	<i>Pst</i> I-GA / <i>Mse</i> I-TCT
	PM15	<i>Pst</i> I-GT / <i>Mse</i> I-GAC
	PM16	<i>Pst</i> I-GT / <i>Mse</i> I-TCT
	PM17	<i>Pst</i> I-AT / <i>Mse</i> I-AAG
25	PM18	<i>Pst</i> I-AT / <i>Mse</i> I-TAG
	PM19	<i>Pst</i> I-GG / <i>Mse</i> I-ATT
	PM20	<i>Pst</i> I-TG / <i>Mse</i> I-AAT
	PM21	<i>Pst</i> I-TG / <i>Mse</i> I-TTT
	PM22	<i>Pst</i> I-TG / <i>Mse</i> I-GCT
30	PM23	<i>Pst</i> I-GT / <i>Mse</i> I-GAA
	PM24	<i>Pst</i> I-AA / <i>Mse</i> I-CTG

27

PM25	<i>Pst</i> I-AC / <i>Mse</i> I-GTG
PM27	<i>Pst</i> I-AA / <i>Mse</i> I-CTA
PM29	<i>Pst</i> I-TA / <i>Mse</i> I-GGA

5

EXAMPLE 3: CONSTRUCTION AND SCREENING OF A TOMATO YAC LIBRARY

Material

10 The tomato line *Lycopersicon esculentum* E22 (Enza Zaden) homozygous for the *Mi* locus, was used as source DNA to construct a YAC library. Protoplasts were isolated from the leaves of *in vitro* shoots which were two to three weeks old as described by Van Daelen *et al* (Plant Mol. Biol. 12, 341-352).

15 Viable protoplasts (concentration of 50 million protoplasts per ml) were collected and mixed with an equal volume of agarose (1%, Seaplaque, FMC Bioproducts, Rockland, Maine, USA) to form a plug. The protoplasts embedded into the plugs were lysed with lysis mix (0.5 M EDTA, 1% N-Laurylsarcosinate and 1 mg/ml proteinase K, pH= 8.0). After lysis, the plugs were stored at 4°C in storage buffer (fresh lysis mix) until used. Approximately 3 million protoplasts per plug, to obtain about 4.5 µg of chromosomal DNA were used for further studies. Plasmid pYAC4
20 containing an unique *Eco*RI cloning site was used as cloning vector and the yeast strain AB1380 was used as a host (Burke *et al*, Science 236, 806-812).

YAC library construction

25 High molecular weight DNA isolation, partial digestion with *Eco*RI in the presence of *Eco*RI methylase, ligation of vector arms to genomic DNA, size selection by pulsed field gel electrophoresis and transformation of the yeast host was performed as described by Burke *et al*, (Science 236, 806-812) and Larin *et al*, (Proc Natl Acad Sci USA 88, 4123-4127).

30 All standard manipulations were carried out as described in Molecular cloning: a laboratory manual by Sambrook *et al*, (Cold Spring Harbor Laboratory Press).

28

3840 clones with a average insert size of 520 kb, which corresponds to 2.2 genome equivalents were finally obtained and the individual clones were stored in 40 96-wells microtiter plates containing 75 µl YPD solution (1% yeast extract, 2% peptone and 2% dextrose).

5

Screening YAC library

To reduce the number of samples handled, the cells of one 96-well microtiter plate were pooled (a platepool) and used for DNA isolation as described by Ross *et al* (Nucleic Acids Res., 19, 6053). The 2.2 genome equivalent tomato YAC library consists of 40 96-wells microtiter wells and as a result DNA of the 40 platepools were screened with the AFLP markers PM10, PM13, PM21 and PM25 using the AFLP protocol as described in Example 2. PM10, PM13, PM21 and PM25 were selected to screen the YAC platepools because these markers do not interfere with the background bands of the yeast strain AB1380. Three positive platepools out of the 40 were identified with these four AFLP markers as shown in Table 2. Subsequently, a secondary screening with the four AFLP markers (PM10, PM13, PM21 and PM25) of the 96 individual YAC clones of each plate was employed to find the correct address of the YAC clones. Three individual YAC clones were identified, designated 1/1084, 1/1172 and 2/1256 (Table 2). Subsequently, the three individual YAC clones were analyzed with the remaining AFLP markers. All of the identified markers PM02 to PM29 were present on one or more these three YAC clones (Table 3). The size of the YAC clone was determined by Pulse-field gel electrophoretic (PFGE) analysis using contour-clamped homogeneous electric field (CHEF; Chu *et al* Science, 235, 1582-1585) and appeared to be 470 kb (1/1084), 570 kb (1/1172), and 500 kb (2/1256) respectively.

23

TABLE 2

	Platepool nr	PM10	PM13	PM21	PM25	YAC detected (size in kb)
5	2	-	-	+	-	YAC 1/1172 (570 kb)
	16	+	+	-	+	YAC 2/1256 (500 kb)
	4	-	+	-	-	YAC 1/1084 (470 kb)

10

TABLE 3

	Marker	1/1172	2/1256	1/1084
15	PM02	-	-	+
	PM07	-	+	-
	PM08	-	+	+
	PM10	-	+	-
	PM11	-	+	-
20	PM13	-	+	+
	PM14	+	+	-
	PM15	-	+	-
	PM16	+	-	-
	PM17	-	+	-
25	PM18	-	+	+
	PM19	-	+	-
	PM20	-	+	-
	PM21	+	-	-
	PM22	-	+	+
30	PM23	-	+	-
	PM24	-	+	-

		30	
PM25	-	+	-
PM27	-	+	-
PM29	-	+	-

5

**EXAMPLE 4: CONSTRUCTION OF A LONG RANGE PHYSICAL MAP OF
THE *Mi* YAC CONTIG AND LOCATION OF THE AFLP
MARKERS**

The 3 YAC clones 1/1172, 2/1256 and 1/1084 were subjected to partial digestion
with increasing concentration of the restriction enzymes *Sfi*I and *Bss*HI. The
samples were fractionated by PFGE, transferred to a Gene Screen Plus membrane
(DuPont NEN, Boston, MA, USA) and assayed by hybridization using end-adjacent
sequence probes according to the protocol for indirect end-label mapping as
described by Burke *et al* (Science 236, 806-812). A physical map of YAC 1/1172,
2/1256 and 1/1084 for the enzymes *Sfi*I and *Bss*HI could be constructed as shown
in Figure 1. The overlap between the various YAC clones was determined by
Southern blot analysis using the obtained restriction fragments as a probe on digest
of the three YAC clones. A YAC contig with a size of 1.4 Mb could be constructed. In
order to isolate the YAC fragments the digests were run on PFGE. Digestion of YAC
1/1172 with *Sfi*I resulted in two fragments (200 Kb and 370 Kb). Digestion of YAC
2/1256 with *Bss*HI resulted in four fragments (40 Kb, 90 Kb, 110 Kb and 260 Kb)
whereas digestion of YAC 1/1084 with *Bss*HI gave two fragments with a size of 70
and 400 kb. As a result the 1.4 Mb YAC contig could be dissected into 8 regions
corresponding to the 8 restriction fragments obtained from the three YAC clones,
covering the complete *Mi* region and adjacent sequences.

To position the various AFLP markers within these 8 regions on the physical map,
the AFLP markers were used as hybridization probes on the partial and complete *Sfi*I
and *Bss*HI digests of YAC clones 1/1172, 2/1256 and 1/1084. Therefore, each
AFLP marker fragment was excised from the dried gel and eluted by means of
diffusion in a buffer containing 0.5 M ammonium acetate, 10 mM magnesium
acetate, 1 mM EDTA (pH=8.0), 0.1% SDS, re-amplified with the corresponding

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unlabelled AFLP primers and, subsequently labelled with ^{32}P according to the random primer method of Feinberg and Vogelstein (Anal. Biochem. 132, 6-10). Each AFLP marker could be assigned to one or more of the eight regions as outlined in Table 4 and Figure 1.

5

TABLE 4

YAC fragment	<i>Mi</i> linked AFLP markers detected by hybridization
10 200 kb <i>Sfi</i> I-fragment 1/1172	-
370 kb <i>Sfi</i> I-fragment 1/1172	PM14, PM16, PM21
260 kb <i>Bss</i> HII-fragment 2/1256	PM10, PM11, PM17, PM19, PM23, PM24, PM29
90 kb <i>Bss</i> HII-fragment 2/1256	PM07, PM27
15 110 kb <i>Bss</i> HII-fragment 2/1256	PM08, PM13, PM14, PM15, PM20, PM22, PM25
40 kb <i>Bss</i> HII-fragment 2/1256	PM18
70 kb <i>Bss</i> HII-fragment 1/1084	PM08, PM13, PM22
400 kb <i>Bss</i> HII-fragment 1/1084	PM02, PM18
20	

EXAMPLE 5: CONSTRUCTION OF A COSMID LIBRARY OF YAC CLONES 1/1172 AND 2/1256

25 Material

The binary cosmid vector pJJ04541 is a derivative of pJJ1881 (Jones *et al*, Transgenic Research 1, 285-297) and is based on plasmid pRK290 containing the tetracyclin resistance gene for selection in *Escherichia coli* and *Agrobacterium tumefaciens*. Into the unique *Eco*RI site of pRK290, T-DNA carrying sequences (LB; left border repeat, RB signifies the right border repeat) that flank

30 - the cos site of bacteriophage lambda

- the neomycin phosphotransferase gene (Beck *et al*, Gene **19**, 327-336) whose expression is driven by the cauliflower mosaic virus 35S promoter sequence (Odell *et al*, Mol Gen Genet **223**, 369-378), and
 - the pBluescript (Stratagene, La Jolla, California, USA) polylinker sequence.
- 5 The size of pJJ04541 amounts 29 kb and is shown schematically in Figure 2. The cloning capacity of this binary cosmid vector, using phage lambda packaging extracts is within the range of 9 to 24 kb.

Library construction

- 10 Total DNA of the *Saccharomyces cerevisiae* strain AB1380 containing YAC 1/1172 and total DNA of the *Saccharomyces cerevisiae* strain AB1380 containing YAC 2/1256 was isolated using zymolyase to make protoplasts according to Green and Olsen (Proc Natl Acad Sci USA **87**, 1213-1217).

- An aliquot of both DNAs was analyzed on PFGE. Both DNA isolates appeared to
15 have a size of ≥ 100 kb.

- Approximately 15 μ g of each DNA was partially digested with *Sau*3A generating molecules with an average size of 15-25 kb. Subsequently, the samples were centrifugated through a 10-35% sucrose gradient for 22 hours, 22.000 rpm at 20°C in a Beckman SW41 rotor. 0.5 ml fractions were collected using a needle pierced
20 through the bottom of the centrifuge tube. An aliquot of these fractions was analyzed on a 0.7% agarose gel. The fractions containing DNA molecules with a size of ≈ 20 kb were pooled and concentrated by ethanol precipitation.

- Subsequently, the cohesive ends were partially filled-in with dATP and dGTP using the strategy of partial filling of 5'-extensions of DNA produced by type II restriction
25 endonuclease as described by Korch (Nucleic Acids Res. **15**, 3199-3220) and Loftus *et al* (Biotechniques **12**, 172-176).

- The binary cosmid vector pJJ04541 was digested completely with *Xho*I and the linear fragment was partially filled-in with dTTP and dCTP as described by Korch (Nucleic Acids Res. **15**, 3199-3220).

- 30 The 20-kb fragments were ligated to the cosmid vector and transduced to *E. coli* strain XL1-Blue MR (Stratagene, La Jolla, California, USA) using phage lambda

33

Gigapack II XL packaging extracts (Stratagene, La Jolla, California, USA) as recommended by the manufacturers. Selection was performed on LB (1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl, pH 7.5) agar plates containing 10 mg/l of tetracyclin. Two banks of approximately 250.000 cosmid clones per bank
5 were made from 2-3 µg of size fractionated yeast DNA of YAC clones 1/1172 and 2/1256 respectively.

Subsequently, these transformants were stored into the wells of microtiter plates (96-wells, 100 µl of LB medium containing 10 mg/l of tetracyclin). Replicas of the 96-well grid of cosmid clones in microtiter plates were stamped onto Gene Screen Plus
10 membrane filters (NEN Dupont) and allowed to grow into colonies on media. Colony hybridization, as described by Sambrook *et al* (in: Molecular cloning: a laboratory manual, 1989, Cold Spring Harbor Laboratory Press), using ³²P-labelled YAC clones 1/1172 and 2/1256 revealed positive cosmids. Of about 10.000 colonies of YAC 1/1172 approximately 200 positive cosmid clones were identified. Of about 20.000
15 colonies of YAC 2/1256 300 positive cosmid clones were identified.

**EXAMPLE 6: FINE MAPPING OF THE *Mi* RESISTANCE GENE SEGMENT
AND POSITIONING OF THE AFLP MARKERS**

20 **Dividing the cosmids in defined regions**

In order to divide the cosmids into seven defined regions, the 200 positive cosmid clones of YAC 1/1172 and the 300 positive cosmid clones of YAC 2/1256 were hybridized with 7 of the 8 restriction fragments (YAC fragments) as outlined in Example 4 (see Table 4 and Figure 1). Positive cosmids for each of the 7 YAC
25 fragments were identified. In addition, cosmids could be identified which reacted positively with the overlapping restriction fragments of the two different YAC clones.

Construction of a cosmid contig of the *Mi* resistance gene segment

In order to construct a cosmid contig of all the positive identified cosmids in the
30 various defined regions restriction fragment amplification was used. Approximately 500 ng of each cosmid was used for template preparation and the primers in the

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amplification of restriction fragments were a *EcoRI*-primer 5'-GACTGCGTACCAATTC-3' having no selective nucleotides and a *MseI*-primer 5'-GATGAGTCCTGAGTAA-3' having no selective nucleotides according to the method as described in Example 2. The *EcoRI*-primer was labelled at the 5' end and all the
5 500 cosmids were amplified using *EcoRI/MseI*-primer set. The DNA fingerprints contained about 8 to 20 amplified fragments. Sets of cosmids containing amplified fragments of identical size were selected from each region and were rerun on polyacrylamide gels as described in Example 2 until a contiguous array of all the amplified fragments throughout the defined regions could be constructed. In addition,
10 the cosmid contig of one region was aligned with the adjacent regions in order to construct a cosmid contig of the *Mi* locus. In this way a cosmid contig of 96 cosmids was constructed spanning the *Mi* locus of approximately 800 kb.

Detailed positioning of the *Mi* linked AFLP markers on the cosmid contig

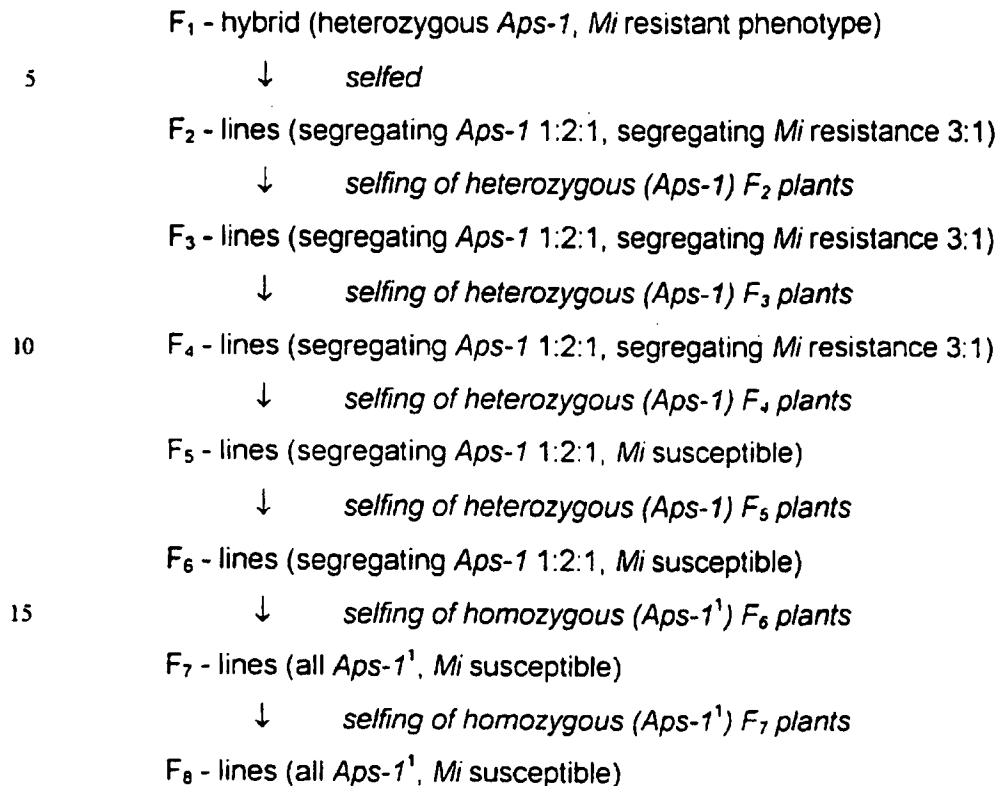
15 In order to position the 20 *Mi* linked AFLP markers on the cosmid contig, the 96 cosmids were digested with *PstI* followed by Southern blot analysis according to Southern, J. Mol. Biol. **98**, 503-515.

The AFLP markers were used as hybridization probes as described in Example 4 on the Southern blot of the 96 *PstI* digests of the cosmids. The exact position of the *Mi*
20 linked AFLP markers, except marker PM02, is outlined in Figure 3A.

EXAMPLE 7: GENETIC ANALYSIS OF *Mi* MUTANTS

A family of mutant tomato lines was made available through Enza Zaden. These lines were derived from a F₁ hybrid heterozygous for the *Mi* resistance gene and
25 heterozygous for the *Aps-1* gene (encoding acid phosphatase-1), which is very closely linked to *Mi* (Stevens and Rick, 1986, in: The Tomato Crop, Atherton & Rudich edit., Chapman and Hall, p. 35-109). Different alleles of the *Aps-1* gene can be determined by isozyme analysis (Vallejos, 1983, in: Isozymes in plant genetics and breeding, Tanksley and Orton edit., part A, Elsevier, Amsterdam, 469-515) The
30 *Aps-1*¹ allele originates from *L. peruvianum* and has been introgressed into several

nematode resistant tomato genotypes by co-segregation with the *Mi* resistance gene. A scheme of these mutant lines is depicted below:



20 In the F_1 , F_2 , F_3 and F_4 lines of this family the presence of the $Aps-1^1$ allele correlates with the *Mi* resistant phenotype, whereas absence of the $Aps-1^1$ allele correlates with the *Mi* susceptible phenotype. In the F_5 and subsequent progenies this correlation is lost: all plants are susceptible to nematodes regardless of the *Aps-1* alleles.

25 Twenty individuals from each F_2 , F_3 , F_4 , F_5 , F_6 , F_7 and F_8 generation were tested for nematode resistance, for presence of the *Aps-1* allele and presence of the *Mi* linked AFLP markers. Nematode testing of seedlings was performed in soil contaminated with root galls of *M. incognita*. The nematode resistance results were as indicated in the above scheme: 3:1 segregation in F_2 , F_3 and F_4 plants and susceptibility in F_5

30 and progeny populations. Most of the *Mi* linked AFLP markers indicated an identical *Mi* genotype as the *Aps-1* isozyme marker. However, 3 of the AFLP markers PM14,

PM16 and PM25 appeared to segregate with the *Mi* phenotype: In most F₅, F₆, F₇ and F₈ plants the *Mi* susceptibility was indicated by the absence of these markers. The AFLP markers PM14, PM16 and PM25 showed a correlation between the AFLP *Mi* genotype and *Mi* phenotype in the mutants. Markers PM14 and PM25 are
5 adjacent on the physical map as shown in Figure 3B, and therefore, it was postulated that the region surrounding these AFLP markers was a good candidate to comprise the *Mi* resistance gene.

**EXAMPLE 8: PHYSICAL MAP OF THE OVERLAPPING COSMID CLONES
10 COMPRISING THE *Mi* RESISTANCE GENE**

The identification of cosmids hybridizing with the *Mi* linked AFLP markers PM14 and PM25 was performed in Example 6. PM14 identifies cosmids Mi-11, Mi-18 and Mi-01 whereas PM25 identifies cosmids Mi-18 and Mi-01.

Subsequently, a small cosmid array around cosmids Mi-11, Mi-18 and Mi-01 was
15 selected from the cosmid contig described in Example 6. A contig of 6 cosmids comprising the 3 identified cosmids and the adjacent cosmids, was selected. These 6 cosmids are Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14. In order to make a physical fine map of these 6 cosmids, the DNA samples of the cosmid contig were digested with *Pst*I followed by electrophoresis on a 0.8%-agarose gel. The physical
20 overlap between the various cosmids could be determined. Combining these data with the data obtained about the detailed positioning of the *Mi* linked AFLP markers on the cosmid contig (see Example 6) a physical fine map with the location of PM14 and PM25 could be constructed as shown in Figure 4. The cosmid contig around the AFLP markers PM14 and PM25 was calculated to be approximately 50 kb.

25

EXAMPLE 9: TRANSFORMATION

Transfer of cosmids to *Agrobacterium tumefaciens*

The cosmid clones Mi-32, Mi-30, Mi-11, Mi-18, Mi-01, Mi-14 and the control cosmid
30 pJJ04541 were introduced in *Agrobacterium tumefaciens* through conjugative transfer in a tri-parental mating with helper strain HB101 (pRK2013) essentially

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according to Deblaere *et al* (Methods in Enzymology **153**, 277-292). *E.coli* were grown in LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl, pH 7.5) supplemented with 5 mg/l tetracyclin at 37°C. The helper strain HB101 (pRK2013) was grown under identical conditions in LB medium supplemented with
5 100 mg/l kanamycin sulphate.

Agrobacterium tumefaciens strain AGL1 (Lazo *et al*, Bio/Technology, **9**, 963-971, 1991) was grown in LB medium supplemented with 100 mg/l carbenicillin at 28°C.

Overnight cultures were diluted 1:100 in LB medium without any antibiotics and after 6 hours of growth, 0.1 ml each of the *Agrobacterium* culture, the helper strain culture
10 and a cosmid strain culture were mixed and plated on LB agar plates without antibiotics. After overnight incubation at 28°C, the mixture was plated on LB medium agar plates containing 100 mg/l carbenicillin and 10 mg/l tetracyclin to screen for transconjugants. Plates were incubated for 3-4 days at 28°C. Two serial passages through selective agar plates were performed to select for single transconjugant
15 *Agrobacterium* colonies.

Characterization of *A. tumefaciens* transconjugants

Small-scale cultures were grown from selected colonies and grown in LB medium containing 10 mg/l tetracyclin. Plasmid DNA was isolated by alkaline lysis using the
20 method as described by Ish-Horowicz *et al* (Nucl. Acids Res. **9**, 2989-2997, 1981), and digested with *Bgl*II using standard techniques. In addition, restriction fragment amplification on miniprep DNA of *A. tumefaciens* was performed using the enzyme combination *Eco*RI/*Mse*I and primers having no selective nucleotide as described in Example 6. Subsequently, the *Bgl*II restriction enzyme pattern as well as the DNA
25 fingerprint of the *A. tumefaciens* transconjugant were compared with those of miniprep DNA of the *E. coli* strain containing the cosmid. Only those *A. tumefaciens* transconjugants harbouring a cosmid with the same DNA pattern as the corresponding *E. coli* culture were used to transform a susceptible tomato line.

38

Transformation of a susceptible tomato line

Seeds of the susceptible tomato line 52201 (Rijk Zwaan, De Lier, The Netherlands) were surface-sterilized in 2% sodium hypochlorite for 10 minutes, rinsed three times in sterile distilled water, and placed on germination medium (consisting of half-strength MS medium according to Murashige and Skoog, Physiol. Plant. 15, 473-497, with 1% (w/v) sucrose and 0.8% agar) in glass jars or polypropylene culture vessels. They were left to germinate for 8 days. Culture conditions were 25°C, a photon flux density of 30 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ and a photoperiod of 16 /24 h.

Transformation of tomato was performed according to Koornneef *et al* (1986), In: Tomato Biotechnology, 169-178, Alan R. Liss, Inc., and is described briefly below. Eight day old cotyledon explants were precultured for 24 hours in Petri dishes containing a feeder layer of *Petunia hybrida* suspension cells plated on MS20 medium (culture medium according to Murashige and Skoog, (1962) Physiol. Plant. 15, 473-497 with 2% (w/v) sucrose and 0.8% agar) supplemented with 10.7 μM α -naphthaleneacetic acid and 4.4 μM 6-benzylaminopurine. The explants were then infected with the diluted overnight culture of *Agrobacterium tumefaciens* containing the cosmid clones Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14 or the cosmid vector pJJ04541 for 5-10 minutes, blotted dry on sterile filter paper and cocultured for 48 hours on the original feeder layer plates. Culture conditions were as described above. Overnight cultures of *Agrobacterium tumefaciens* were diluted in liquid MS20 medium (medium according to Murashige and Skoog (1962) with 2% (w/v) sucrose, pH 5.7) to an O.D.₆₀₀ of 0.8.

Following the cocultivation, the cotyledon explants were transferred to Petri dishes with selective medium consisting of MS20 supplemented with 4.56 μM zeatin, 67.3 μM vancomycin, 418.9 μM cefotaxime and 171.6 μM kanamycin sulphate, and cultured under the culture conditions described above. The explants were subcultured every 3 weeks onto fresh medium. Emerging shoots were dissected from the underlying callus and transferred to glass jars with selective medium without zeatin to form roots. The formation of roots in a medium containing kanamycin sulphate was regarded as an indication of the transgenic nature of the shoot in question. Truly transgenic regenerants were propagated *in vitro* by subculturing the

apical meristem and auxiliary buds into glass jars with fresh selective medium without zeatin.

EXAMPLE 10: COMPLEMENTATION ANALYSIS

5

Identification of cosmids with the *Mi* resistance gene by screening for resistance in roots of transformed plants

Roots of *in vitro* grown transformed R_0 plants have been subjected to the disease assay as described in Example 1. From each transformant two root explants have been assayed. In total 72 R_0 plants of 7 different cosmid transformations have been tested; 6 cosmids carrying tomato insert DNA and one cosmid, pJJ04541, is without tomato insert DNA. The results are shown in Table 1. Sixty three transgenic R_0 plants appeared susceptible, because galls had been formed on at least one of the two root cultures. Nine R_0 plants scored resistant, because no galls could be found on the root cultures. Seven resistant plants had been derived from transformation with cosmid Mi-11, while two resistant plants had been derived with cosmid Mi-18, that is overlapping for a great part with cosmid Mi-11. The cosmids Mi-11 and Mi-18 were used for further molecular analysis.

20

TABLE 1

Cosmid	R_0 plants	
	Resistant	Susceptible
Mi-32	0	8
Mi-30	0	11
Mi-11	7	4
Mi-18	2	8
Mi-01	0	10
Mi-14	0	15
pJJ04541	0	7

25

30

Molecular analysis of the transformed plants with a resistant phenotype

To demonstrate that the resistant phenotype of transgenic R_0 plants, which had been with the overlapping cosmids Mi-11 and Mi-18, is determined by the genomic insert present in the various cosmids, an AFLP analysis with the AFLP marker PM14 was performed. Selective restriction fragment amplification was performed with the primer combination identifying marker PM14 for the R_0 plants transformed with cosmids Mi-11 and Mi-18. The DNA fingerprints obtained showed the presence of the marker PM14 in the resistant plants indicating that the genomic insert present in cosmids Mi-11 and Mi-18 is also present in the R_0 plants and that the two identified overlapping cosmids Mi-11 and Mi-18 comprise the *Mi* resistance gene.

The inserts in cosmids Mi-11 and Mi-18 and the inserts in the adjacent cosmids Mi-32, Mi-30 on one side and cosmids Mi-01 and Mi-14 on the other side, were further characterized. The DNA region comprising the *Mi* resistance gene based on the overlap between the cosmids Mi-11 and Mi-18, was estimated at approximately 16-18 kb. Based on the susceptibility of the R_0 plants having the insert present in cosmid Mi-30, this region could be narrowed down to approximately 12 kb. A DNA segment comprising the *Mi* resistance gene, corresponding to the region flanked by the right ends of cosmids Mi-30 and Mi-11, was sequenced (see Figure 4).

EXAMPLE 11: NUCLEOTIDE SEQUENCE AND DEDUCED AMINO ACID SEQUENCE OF THE *Mi* RESISTANCE GENE FROM TOMATO

Subcloning of the overlapping DNA segment

To determine the sequence of the overlapping DNA segment in cosmids Mi-11 and Mi-18 containing the *Mi* resistance gene, a set of random subclones with a insert size of approximately 2 kb were generated. 7.5 μ g of CsCl purified DNA of cosmids Mi-11 and Mi-18 was sheared for 10 seconds at 4°C at 15% probe power (in 40 μ l 10mM Tris-acetate, 10mM Mg-acetate and 50mM K-acetate) using a Misonix (Misonix Inc., Farmingdale, NY, USA) sonicator (type XL2020) with a water filled cup horn (type 431A). Subsequently, the DNA was heated for 10 minutes at 60°C and

cooled to room temperature. The ends of the DNA fragments were repaired by adding 10 μ l of a repair mixture (10mM Tris-acetate, 10mM Mg-acetate, 50 mM K-acetate, 10U Klenow DNA polymerase, 10U T₄DNA polymerase and 2 mM of all 4 dNTP's) and followed by incubation for 30 minutes at 20°C. The sheared DNA was
5 separated by electrophoresis on 1% Seakem GTG agarose gel (FMC Bio Products, Rockland, ME, USA). The fraction with a size of 1.8-2.2 kb was excised from the gel and subsequently the gel slice was digested with β -agarase I according to the protocol of the manufacturer (New England Biolabs Inc, Beverly, MA, USA) and the DNA was precipitated.

10 A modified pUC19 vector (designated pStuc) was used to clone the 1.8-2.2 kb fraction. In this vector the BamHI/Sall fragment of pUC19 was replaced by a DNA fragment containing a StuI, SpeI and Sall restriction site using two oligonucleotide primers and standard cloning techniques as described by Sambrook *et al.* (in: Molecular cloning: a laboratory manual, 1989, Cold Spring Harbor Laboratory
15 Press). The 1.8-2.2 kb fraction was ligated at 16°C in the a StuI digested and dephosphorylated pStuc vector. The ligation mixture was subsequently transformed to Epicurian Coli XL2-Blue MRF' ultracompetent cells (Stratagene, La Jolla, CA, USA). Individual colonies were grown and stored in 384-wells microtiter plates (100 μ l of LB medium containing 100 mg/l of carbenicillin).

20 To isolate clones representing the overlapping DNA region in cosmids Mi-11 and Mi-18 containing the *Mi* resistance gene, the 8.6 and 4.5 kb PstI fragment of cosmid clone Mi-18 (see Figure 4) as well as the AFLP marker PM14 were used as hybridization probes in colony hybridizations. Therefore, replicas of the 384-well grid of clones in microtiter plates were stamped onto Gene Screen Plus membrane filters
25 (DuPont NEN, Boston, MA, USA) and allowed to grow into colonies on media.

Eighty four positive clones were used to isolate plasmid DNA using the alkaline lysis method as described by Ish-Horowicz *et al.* 1981, Nucl. Acids Res. 9, 2989-2997.

Sequence analysis

30 The ABI PRISM dye terminator cycle sequencing ready reaction kit was used to perform sequencing reactions in a Gene-Amp PCR system Model 9600 (Perkin-

42

Elmer, Foster City, CA, USA). Standard M13 forward and reverse primers were used. The reaction products were analyzed on 48 cm gels of an ABI Prism 377. The DNA sequence of 84 selected clones was determined using the standard forward and reverse sequencing primers. Sequence assembly and analysis was done with
5 the 1994 version of the STADEN sequence analysis program (Dear and Staden, 1991, Nucl. Acids Res. 19, 3907-3911). A contiguous DNA sequence of approximately 9.9 kb nucleotides could be formed and is shown in Figure 5. A large open reading frame of 3621 nucleotides (ORF2) encoding a truncated polypeptide of 1206 amino acids (figure 7B) could be deduced.

10

EXAMPLE 12: ROOT-KNOT NEMATODE INFECTION: SOIL INOCULATION

Soil infected with the root-knot nematode *Meloidogyne incognita* had been
15 prepared as follows: root systems of heavily infected tomato plants with a high number of galls (or root-knots), were cut into pieces and mixed through fresh soil.

Seeds were sown or small rooted plantlets were transferred into the infected soil. The plants were grown in a greenhouse at a temperature of 25°C. After 4 to 8
20 weeks, the plants were carefully pulled out of the soil and the roots were rinsed in water in order to remove the adhering soil. The level of infection was determined by counting the number of galls formed.

Plants were considered to be resistant when three galls or less were visible on the roots. Plants were considered susceptible when more than three galls were
25 formed on the root system.

EXAMPLE 13: COMPLEMENTATION ANALYSIS

Identification of cosmids with the *Mi* resistance gene by screening for resistance
30 in the selfed progenies of transformed plants

The primary regenerants (R_0 generation) of the transformation experiments were grown in the greenhouse for seed set. For each cosmid, ten to fifteen regenerants were grown and R_1 seeds were harvested. R_1 lines of at least seven R_0 plants of each cosmid were tested for resistance against *Meloidogyne*
5 *incognita* in order to identify cosmids with the resistance gene. Twenty to 30 seedlings or plantlets of each R_1 line were inoculated and evaluated as described in Example 12.

In total 63 R_1 lines of 7 different cosmid transformations have been tested; 6 cosmids carrying tomato insert DNA and one cosmid, pJJ04541, without tomato
10 insert DNA. The results are shown in Table 2. Fifty-four transgenic R_0 plants appeared to be susceptible, because galls had been formed on the root systems of all tested R_1 -plants. Nine R_0 plants are considered resistant, because at least half of the plants of each R_1 line had three or less galls. One R_1 line was completely resistant, six R_1 lines segregated in a ratio of about 3:1 (resistant to
15 susceptible plantlets), and the progenies of two R_0 plants segregated 1:1. All the nine resistant R_0 plants had been derived from transformations with cosmid Mi-11.

Additional genetic evidence for the presence of the *Mi* resistance gene on cosmid Mi-11 was obtained in the next generation. Resistant R_1 plants were
20 selfed. Fourteen of the resulting R_2 lines, which originated from four different R_0 plants, were tested for resistance against *M. incognita*. Twenty to thirty seedlings of each R_2 line were inoculated and evaluated as described in Example 12. The results are shown in Table 3. Five R_2 lines were completely resistant, indicating that the parental R_1 plants were homozygous for the *Mi* resistance gene. Eight R_2
25 lines segregated in a ratio of 3:1, indicating that their parental R_1 plants were heterozygous for the *Mi* resistance gene. One R_2 line was segregating in a ratio of about 1:1, and none of the tested lines appeared to be completely susceptible. These results prove that the selected R_1 plants, which are derived from several plants transformed with cosmid Mi-11, contain the functional *Mi* resistance gene.

TABLE 2

Cosmid	Number of R ₁ -lines of independent R ₀ plants tested				
	Total	Segregation ratio R:S (resistant to susceptible)			
		1:0	3:1	1:1	0:1
Mi-32	7	0	0	0	7
Mi-30	9	0	0	0	9
Mi-11	9	1	6	2	0
Mi-18	8	0	0	0	8
Mi-01	10	0	0	0	10
Mi-14	9	0	0	0	9
pKK04541	11	0	0	0	11

TABLE 3

Cosmid	Number of R ₂ -lines of independent R ₁ plants tested				
	Total	Segregation ratio R:S (resistant to susceptible)			
		1:0	3:1	1:1	0:1
Mi-11	14	5	8	1	0

5

EXAMPLE 14: POTATO APHID INFECTION ASSAY

Small tomato plants (4 weeks old) were inoculated with the potato aphid (*Macrosiphum euphorbiae*) by placing five to eight female aphids on the leaves.

10 The plants were grown in the greenhouse at a temperature of 18° to 20°C. After one to two weeks the level of resistance was determined by counting the number of newly born aphids.

Plants were considered to be resistant when no living aphids were present on the stem or leaves. plants were susceptible when newly born aphids were
 15 present.

EXAMPLE 15: COMPLEMENTATION ANALYSIS

Identification of cosmids with the *Meu-1* resistance gene by screening for
 5 resistance in the selfed progenies of transformed plants.

A subset of the R_1 lines obtained in Example 13 was tested for resistance against
Macrosiphum euphorbiae in order to identify cosmids with the *Meu-1* resistance
 gene. Ten to fifteen plantlets of each R_1 line were inoculated and evaluated as
 10 described in Example 14. In total 41 R_1 lines of 7 different cosmid
 transformations have been tested; 6 cosmids carrying tomato insert DNA and
 one cosmid, pJJ04541, without tomato insert DNA. The results are shown in
 Table 4. Thirty-six transgenic R_0 plants are considered susceptible, because
 dozens of aphids were proliferating on all or most plants of each R_1 line. Five R_0
 15 plants are resistant, because at least half of the plants of each R_1 line were
 without living aphids. All these five resistant R_0 plants had been transformed with
 cosmid Mi-11.

The obtained results strongly indicate that the R_0 plants which are derived from
 transformations with cosmid Mi-11, contain a functional *Meu-1* resistance gene.

20

TABLE 4

Cosmid	Number of R_1 -lines of independent R_0 plants tested				
	Total	Segregation ratio R:S (resistant to susceptible)			
		1:0	3:1	1:1	0:1
Mi-32	4	0	0	0	4
Mi-30	7	0	0	0	7
Mi-11	7	1	2	2	2
Mi-18	7	0	0	0	7
Mi-01	6	0	0	0	6
Mi-14	5	0	0	0	5
pJJ04541	5	0	0	0	5

Additional genetic evidence for the presence of the *Meu-1* resistance gene on cosmid Mi-11 was obtained in the next generation. Twenty-four R_2 lines that had been obtained from selfings of nematode resistant R_1 plants (see Example 13), which originated from nine different R_0 plants, were tested for resistance against *M. euphorbiae*. Eleven to fifteen seedlings of each R_2 line were inoculated and evaluated as described in Example 14. The results are shown in Table 5. One R_2 line segregated in a ratio of 3:1 and eight R_2 lines were segregating in a ratio of about 1:1. In these nine lines the potato aphid resistance phenotype is clearly visible. Five R_2 lines appeared to be completely susceptible. The remaining ten R_2 lines scored intermediate: they segregated in a ratio of about 1:3. These results indicate that several R_1 plants, which are resistant to *Meloidogyne incognita* and which are derived from R_0 plants transformed with cosmid Mi-11, have a functional *Meu-1* resistance gene.

In addition, eight R_1 BC lines that were obtained from nematode resistant R_1 plants backcrossed with susceptible tomato line 52201 were tested for resistance against *M. euphorbiae*, in order to confirm inheritance of the introgressed *Meu-1* resistance gene. Twelve to fifteen seedlings of each R_1 BC line were inoculated and evaluated as described in Example 14. The results are shown in Table 6.

The segregation ratios shown in Table 5 and Table 6 only serve to illustrate the inheritance of the resistance phenotype.

TABLE 5

Cosmid	Number of R_2 -lines of independent R_1 plants tested					
	Total	Segregation ratio R:S (resistant and susceptible)				
		1:0	3:1	1:1	1:3	0:1
Mi-11	24	0	1	8	10	5

TABLE 6

Cosmid	Number of R ₁ BC-lines of independent R ₁ plants tested					
	Total	1:0	3:1	1:1	1:3	0:1
Mi-11	8	0	1	5	2	0

EXAMPLE 16: TRANSCRIPT MAPPING

Transcript mapping studies were performed to map the 5' and 3' end of the *Mi*-resistance gene and to determine whether the *Mi* resistance gene contains any introns. The polymerase chain reaction to amplify parts of the transcripts from the *Mi* resistance gene was used for this purpose.

Total RNA from leaf tissue of the resistant tomato cultivar E22 was isolated according to the hot phenol method as described by Sambrook *et al* (in: Molecular cloning: a laboratory manual, 1989, Cold Spring Harbor Laboratory Press). Poly A+ RNA was isolated using biotinylated oligo(dT) bound to Dynabeads M-280 Streptavidin (DYNAL A.S., Oslo, Norway) according to the instructions of the manufacturer. A cDNA library was constructed using the Superscript Rnase H Reverse Transcriptase cDNA kit from Life technologies, Inc. Gaithersburg, MD, USA and the protocol supplied by the manufacturer.

5' and 3' RACE products were obtained using the Marathon cDNA amplification kit from Clontech (Paolo Alto, CA, USA). The primers used were designed based on the genomic *Mi*-sequence, and especially on the 5' end of the coding sequence of ORF2. Subsequently, the various 5' and 3'-RACE fragments were cloned into the TA cloning vector pCRII (Invitrogen Corporation, San Diego, CA, USA) and sequenced using standard protocols. The nucleotide sequences obtained were aligned with the 9.9 kb genomic sequence and two intron sequences could be deduced for the 5' end of the *Mi* resistance gene. One intron of 1306 nucleotides was located from nucleotide position 1936 to 3241 and the second one from nucleotide position 3305 to 3379 (Figure 5).

The largest Mi-transcript detected with the Marathon cDNA amplification kit maps at nucleotide position 1880. Hence, we conclude that the Mi transcriptional initiation site is positioned at or upstream of nucleotide 1880. The first ATG codon that could be detected within the 5' cDNA was located at nucleotide position 3263, 52 nucleotides upstream of the second intron, and a large open reading frame (ORF1) encoding a polypeptide of 1257 amino acids could be deduced and is shown in Figure 7A. As a result, this second intron is located between amino acid 14 and 15 of the *Mi*-resistance gene product.

10 **EXAMPLE 17: PCR ANALYSIS OF MI-11 AND MI-18 TRANSFORMED PLANTS**

Data obtained from complementation analysis in roots of transformed plants (Example 10) indicated that the *Mi* resistance gene was located on a DNA segment overlapping between cosmids Mi-11 and Mi-18, excluding the DNA segment corresponding to cosmid Mi-30, transformants of which were all susceptible. This region was estimated to be about 12 kb. However, in complementation analysis on the selfed progenies of transformed plants, only cosmid Mi-11 transformed plants scored resistant (Examples 13 and 15). To address the question why Mi-18 transformed plants scored susceptible, a PCR analysis on the presence or absence of the putative Mi-ORF in transformed Mi-11 and Mi-18 plants was performed.

The following DNA samples have been analysed:

1. YAC clone 2/1256.
- 25 2-3. Cosmid Mi-11 in *E. coli* and in *A. tumefaciens*, respectively.
- 4-5. Cosmid Mi-18 in *E. coli* and in *A. tumefaciens*, respectively.
6. Tomato line E22 (resistant).
7. Tomato line 52201 (susceptible).
- 8-12. Five plants transformed with cosmid Mi-11.
- 30 13-17. Five plants transformed with cosmid Mi-18.

The DNA was digested with *Pst*I and *Pst*I-adaptors were ligated. Subsequently, a PCR analysis was performed with a primer identifying the *Pst*I site and three additional selective nucleotides or marker PM14 and various PCR primers located upstream of PM14 using the enzym *rTh* polymerase (Gene Amp XL PCR
5 kit; Perkin Elmer). The products generated varied in size from 443 to 6110 bp and encompass the complete PM14 upstream region of the putative Mi-ORF (see Figure 6).

It appeared that all templates generated PCR products of the expected size with the exception of the five plants transformed with cosmid Mi-18. Only the smallest
10 PCR product (443 bp) was formed. These data indicate that almost the complete PM14 upstream region was not present in plants transformed with cosmid Mi-18. These deletions do not occur with cosmid Mi-18 present in *E. coli* or *A. tumefaciens* but occur only in transformed plants. Hence, we conclude that these deletions are responsible for the susceptible phenotype to *Meloidogyne incognita*
15 and/or *Macrosiphum euphorbiae* of Mi-18 transformed plants.

EXAMPLE 18: NUCLEOTIDE SEQUENCE OF COSMID MI-11

The observation that only plants transformed with cosmid Mi-11 showed a
20 resistant phenotype might indicate that additional open reading frames present on Mi-11 could be candidates to encode for resistance against nematodes and/or aphids. Therefore, the nucleotide sequence of the region upstream of the postulated ORF1 was determined to identify additional open reading frames.

A set of random subclones with an insert size of 2 kb were isolated using the 2.1,
25 4.7 and 2.9 kb *Pst*I fragment of cosmid clone Mi-11 as hybridization probes in colony hybridization essentially as described in Example 11.

Fourty nine positive clones were used to determine the DNA sequence using the standard forward and reverse sequencing primers. Sequence assembly and analysis was performed as described in Example 11.

30 Three contiguous DNA stretches with sizes of 5618 bp (con25), 898 bp (con10) and 2495 bp (con62) could be deduced. The gaps between these DNA stretches

and the 9870 bp DNA sequence containing the putative Mi-ORF (Figure 6) was calculated using PCR and varied between 50-200 bp.

The three determined contigs (con25, con10 and con62) were analysed for the distribution of stop codons in all six possible frames. No significant ORF frames
5 with a size of or superior to 120 amino acids could be postulated. In addition, no DNA homology with the putative ORF1 was detected. Hence, the only significant ORF present on cosmid Mi-11 was ORF1 as described in Figure 5. Based on these results, it can be concluded that the polynucleotide encoded by ORF1 confers resistance to nematodes as well as to aphids and, hence, that the *Mi*-
10 resistance gene and the *Meu-1* resistance gene are referring to the same coding sequence as depicted in figure 5.

EXAMPLE 19: TRANSFORMATION OF TOBACCO AND COMPLEMENTATION ANALYSES

15

Transformation of tobacco

The tobacco cultivar Petit Havana, type SR1, was transformed with cosmid Mi-11 or the cosmid vector pJJ04541 using the protocol as described by Horsch *et al.* (Science 227, 1229-1231, 1985).

20

Complementation analysis: screening for nematode resistance in root cultures of transformed tobacco plants

Roots of *in vitro* grown transformed R₀ plants of tobacco have been subjected to the disease assay as described in Example 1. From each of the 31 transformants
25 two or more root explants have been assayed. In addition, all 17 Mi-11 transformants have been analyzed by PCR for the presence of the putative Mi ORF1 by screening for an internal fragment with a size of 823 base pairs (ranging from nucleotide position 4824 to 5646, see Figure 5). Simple PCR primers for the fragment were deduced from the sequence as shown in Figure 5.
30 The primers used have the following sequences:

51

primer S21: 5'-CCAAGGACAGAGGTCTAATCG-3'

primer S22: 5'-TTGAGGTGATGTGGTAAATGG-3'

Primer S21 targets the sequence from nucleotide position 4824 to 4844 and
 5 primer S22 targets the sequence from nucleotide position 5626 to 5646 (see
 Figure 5).

The results of the *in vitro* disease assay and of the PCR analysis (presence "+"
 or absence "-" of the internal PCR fragment) are shown in Table 7. "Mi-11"
 10 represents transformed plants comprising the putative Mi ORF1 and "Mi-11Δ"
 represents those transformed plants having a deletion in the putative Mi ORF1,
 as determined by the PCR analysis (described above). Twenty-nine R₀
 transformants were susceptible, because galls had been formed on at least one
 of the tested root cultures. Generally, the rate of gall formation on tobacco roots
 15 is slightly lower than on susceptible tomato roots. Two R₀ plants scored resistant
 to *Meloidogyne incognita*, because no galls could be found on the root cultures.
 Both resistant plants were transformed with cosmid Mi-11 comprising the internal
 PCR fragment indicating the presence of the *Mi* resistance gene.

20

TABLE 7

Genotype	PCR fragment	R ₀ plants	
		Resistant	Susceptible
Mi-11	+	2	7
Mi-11Δ	-	0	8
pJJ04541	-	0	14

30

S2

Complementation analysis: screening for aphid resistance in cuttings of transformed tobacco plants

Rooted cuttings of transformed R₀ plants of tobacco were inoculated and evaluated as described in Example 14. From each of the 23 transformants two or three cuttings have been assayed for resistance against *Macrosiphum euphorbiae*. The results of the infection assay and the PCR analysis (as described above) are shown in Table 8. Twenty-one R₀ plants are considered susceptible, because several living aphids were counted on at least one of the tested cuttings. In general, the level of proliferation of the aphids on tobacco is low compared with the proliferation on susceptible tomato plants. Two R₀ plants scored resistant, because all cuttings of these plants were without living aphids. The aphid resistant plants were transformed with cosmid Mi-11, comprising the *Mi* resistance gene, as indicated by the presence of the internal PCR fragment.

TABLE 8

Genotype	PCR fragment	R ₀ plants	
		Resistant	Susceptible
Mi-11	+	2	3
Mi-11Δ	-	0	6
pJJ04541	-	0	12

53

**EXAMPLE 20 TRANSFORMATION OF POTATO AND
COMPLEMENTATION ANALYSES****Transformation of potato**

- 5 The potato variety Diamant (Cebeco Zaden B.V., Vlijmen, The Netherlands) was used for transformation. Internode explants of *in vitro* grown plants were transformed with cosmid Mi-11 or the cosmid vector pJJ04541 using the protocol as described by Ooms *et al.* (Theor. Appl. Genet. **73**, 744-750).
- 10 **Complementation analysis: screening for nematode resistance in root cultures of transformed plants**
- Roots of *in vitro* grown transformed R₀ plants of potato have been subjected to the disease assay as described in Example 1. From each of the 31 transformants at least two root explants have been assayed. In addition, all 26 Mi-11
- 15 transformants have been analyzed by PCR using primers S21 and S22 as described in Example 19. The results of the *in vitro* disease assay and of the PCR analysis (presence "+" or absence "-" of the internal PCR fragment) are shown in Table 9. "Mi-11" represents transformed plants comprising the putative Mi ORF1 and "Mi-11Δ" represents those transformed plants having a deletion in
- 20 the putative Mi ORF1, as determined by the PCR analysis (described above). Twenty-eight R₀ transformants were susceptible, because galls had been formed on at least one of the root cultures. Generally, the rate of gall formation on potato roots is lower than on susceptible tomato roots. Three R₀ plants scored resistant to *Meloidogyne incognita*, because no galls could be found on the root cultures.
- 25 All these resistant plants were transformed with cosmid Mi-11 comprising the internal PCR fragment indicating the presence of the *Mi* resistance gene.

54

TABLE 9

5	Genotype	PCR fragment	R ₀ plants	
			Resistant	Susceptible
	Mi-11	+	3	17
	Mi-11Δ	-	0	6
	pJJ04541	-	0	5

Complementation analysis: screening for nematode resistance in cuttings of transformed plants

Rooted cuttings of Mi-11 transformed R₀ plants of potato have been subjected to the disease assay as described in Example 12. From each of the 19 transformants one to three cuttings have been assayed for resistance against *Meloidogyne incognita*. The results are shown in Table 10. In addition, 36 rooted cuttings of non-transformed potato plants (variety Diamant) were assayed (as susceptible controls) and were all susceptible. One R₀ plant scored resistant to *Meloidogyne incognita*, because no galls could be found on the root system.

TABLE 10

25	Genotype	PCR fragment	R ₀ plants	
			Resistant	Susceptible
	Mi-11	+	1	12
	Mi-11Δ	-	0	6
30	Non-transf. control	-	0	1

55

CLAIMS

1. A nucleic acid whose DNA sequence is the DNA of figure 5 or part thereof or a DNA sequence homologous to the DNA sequence of figure 5.
2. The nucleic acid of claim 1 which is capable, when transferred to a host plant, which is susceptible to a plant pathogen, of rendering said host plant resistant to said plant pathogen.
3. A nucleic acid sequence which is a cDNA corresponding to a nucleic acid whose DNA sequence is at least part of the DNA sequence provided in figure 5.
4. The nucleic acid of claim 1 or 3, which, when transferred to a host plant is capable of rendering it resistant to nematodes.
5. The nucleic acid of claim 1 or 3, which, when transferred to a host plant is capable of rendering it resistant to aphids.
6. The nucleic acid of claim 1 or 3, which, when transferred to a host plant is capable of rendering it resistant to nematodes and aphids.
7. A nucleic acid wherein said DNA sequence corresponds to a sequence starting at nucleotide 3263 and ending at nucleotide 7111 of the sequence of figure 5 or any DNA sequence homologous thereto.
8. A nucleic acid wherein said DNA sequence corresponds to a promoter sequence located 5' upstream of nucleotide 3263 or any DNA sequence homologous thereto.
9. A nucleic acid of claim 1 or 3 wherein said DNA sequence corresponds to at least part of the genomic insert present in cosmid Mi-11, or any DNA sequence homologous thereto.
10. A recombinant DNA construct comprising a nucleic acid according to any of claims 1-9.
11. A recombinant DNA construct of claim 10 in which said nucleic acid is under control of a promoter which is functional in a plant cell, said promoter being either endogenous or exogenous to said plant cell, and effective to control the transcription of said DNA sequence in such plant cells.

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12. A recombinant DNA construct of claim 11 in which said promoter corresponds to a promoter sequence located 5' upstream of nucleotide 3263 as provided in figure 5, or any DNA sequence homologous thereto.

13. A vector suitable for transforming plant cells comprising a DNA
5 construct according to any of claims 10-12.

14. Plasmid pKGMi-11 as deposited under number CBS 822.96.

15. Plasmid pKGMi-18 as deposited under number CBS 821.96.

16. Bacterial cells comprising a vector or plasmid according to any of
claims 13-15.

10 17. Recombinant plant genome comprising, incorporated therein, a DNA
construct according to any of claims 10-12.

18. Plant cells comprising a DNA construct according to any of claims 10-
12.

19. Plant comprising plant cells according to claim 18.

15 20. Plant according to claim 19 which has a reduced susceptibility to
nematodes.

21. Plant according to claim 20 wherein said nematode is a root-knot
nematode, especially *Meloidogyne incognita*.

22. Plant according to claim 19 which has a reduced susceptibility to
20 aphids, especially *Macrosiphum euphorbiae*.

23. Seed comprising a DNA construct according to any of claims 10-12.

24. The recombinant plant genome of claim 17, in a plant cellular
environment.

25 25. Process for obtaining plants having reduced susceptibility to a
pathogen, comprising the following steps:

- i) inserting into the genome of a plant cell a DNA construct according to
any of claims 10-12,
- ii) obtaining transformed plant cells,
- iii) regenerating from said transformed plant cells genetically transformed
30 plants, and

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iv) optionally, propagating said plants.

26. Process according to claim 25 wherein said pathogen is a nematode, and preferably a root-knot nematode, especially *Meloidogyne incognita*.

27. Process according to claim 25 wherein said pathogen is an aphid, and preferably *Macrosiphum euphorbiae*.

28. Process for protecting plants in cultivation against pathogen infection, which comprises:

i) providing the genome of plants with a DNA construct according to any of claims 10-12, and

10 ii) growing said plants.

29. Process for isolating a nucleic acid according to claim 1-6, comprising the following steps:

i) screening a genomic or cDNA library of a plant with a DNA sequence according to claim 1-9,

15 ii) identifying positive clones which hybridize to said DNA sequence,

iii) isolating said positive clones.

30. The process of claim 29 wherein said library originates from a first plant and the DNA sequence belongs to a second plant.

31. Process of selective restriction fragment amplification for identifying a nucleic acid according to claim 1-9 using primer combinations identifying at least one of the AFLP markers PM02 to PM29 as depicted in Table 3.

32. The process of claim 31 wherein said primer combination identifies AFLP marker PM14.

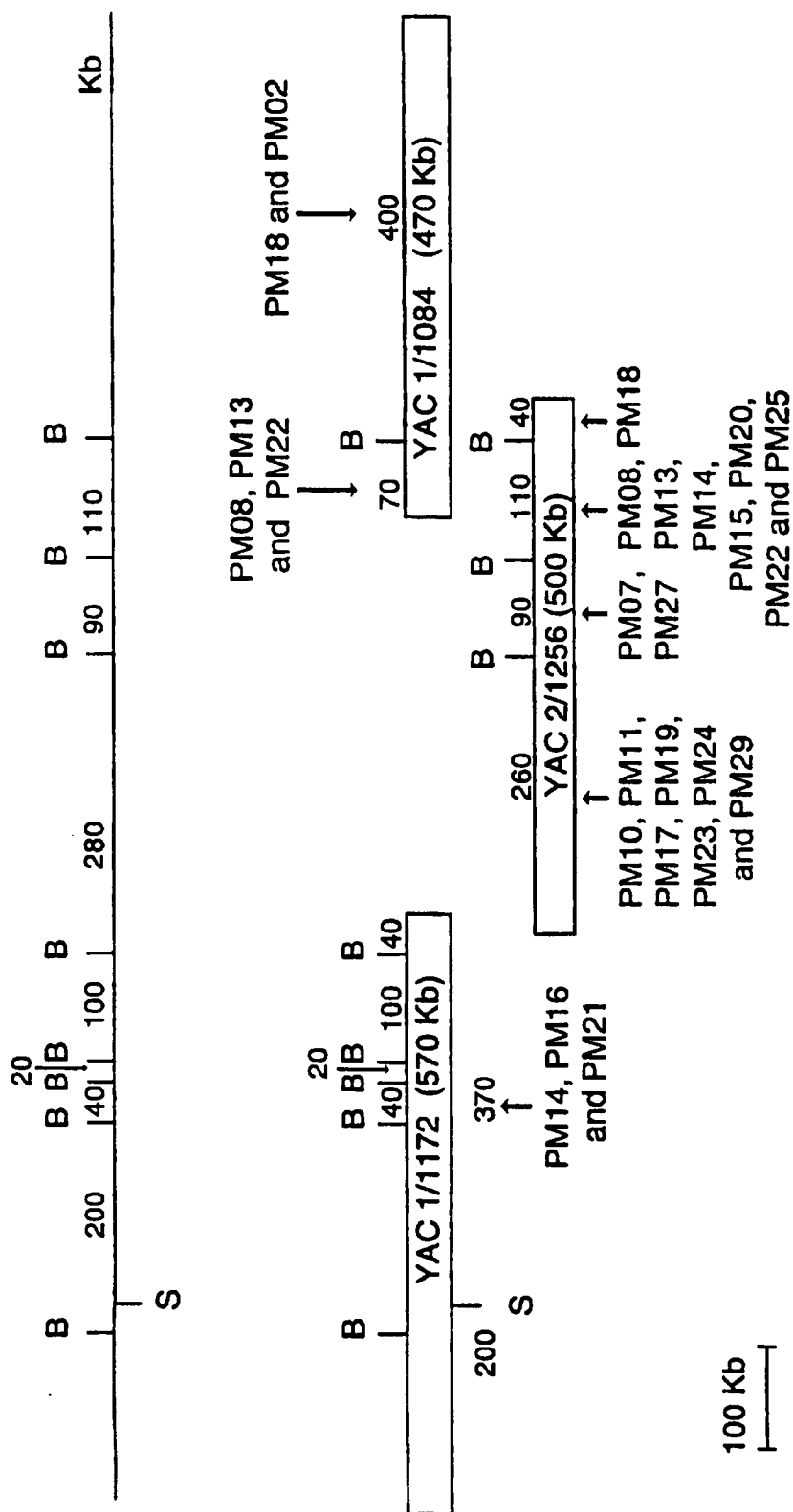
33. An oligonucleotide comprising a DNA sequence which corresponds to at least part of the nucleic acid according to claims 1-9.

34. The oligonucleotide of claim 33, which is of a size sufficient to hybridize selectively to the DNA sequence of any of claims 1 to 9 under stringent hybridization conditions.

35. An oligonucleotide according to claim 34 wherein said DNA sequence corresponds to the sequence starting at nucleotide 6921 and ending at nucleotide 7034.

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36. An oligonucleotide according to claim 35 wherein said DNA sequence is located at the 3'end, and preferably corresponds to the sequence 5'TGCAGGA-3', which can prime the synthesis of DNA.
37. An oligonucleotide according to claim 35 wherein said DNA sequence
5 is located at the 3'end, and preferably corresponds to the sequence 5'-TAATCT-3' which can prime the synthesis of DNA.
38. A primer combination comprising a first oligonucleotide according to claim 36 and a second oligonucleotide according to claim 37.
39. Diagnostic kit comprising at least one oligonucleotide according to any
10 of claims 33-37.
40. Diagnostic kit comprising a primer combination according to claim 38.
41. Process for detecting the presence or absence of a DNA sequence according to claim 1-9, particularly in a plant DNA using a diagnostic kit according to claim 39 or 40.
- 15 42. A polypeptide which is the expression product of a nucleic acid of recombinant DNA according to anyone of claims 1 to 13.
43. A polypeptide having an amino acid sequence having the sequence provided in figure 7A or coded by the corresponding homologous sequence according to anyone of claims 1 to 3.
- 20 44. A RNA having a ribonucleic acid sequence of a transcript of part or all of the DNA sequence of anyone of claims 1 to 3.



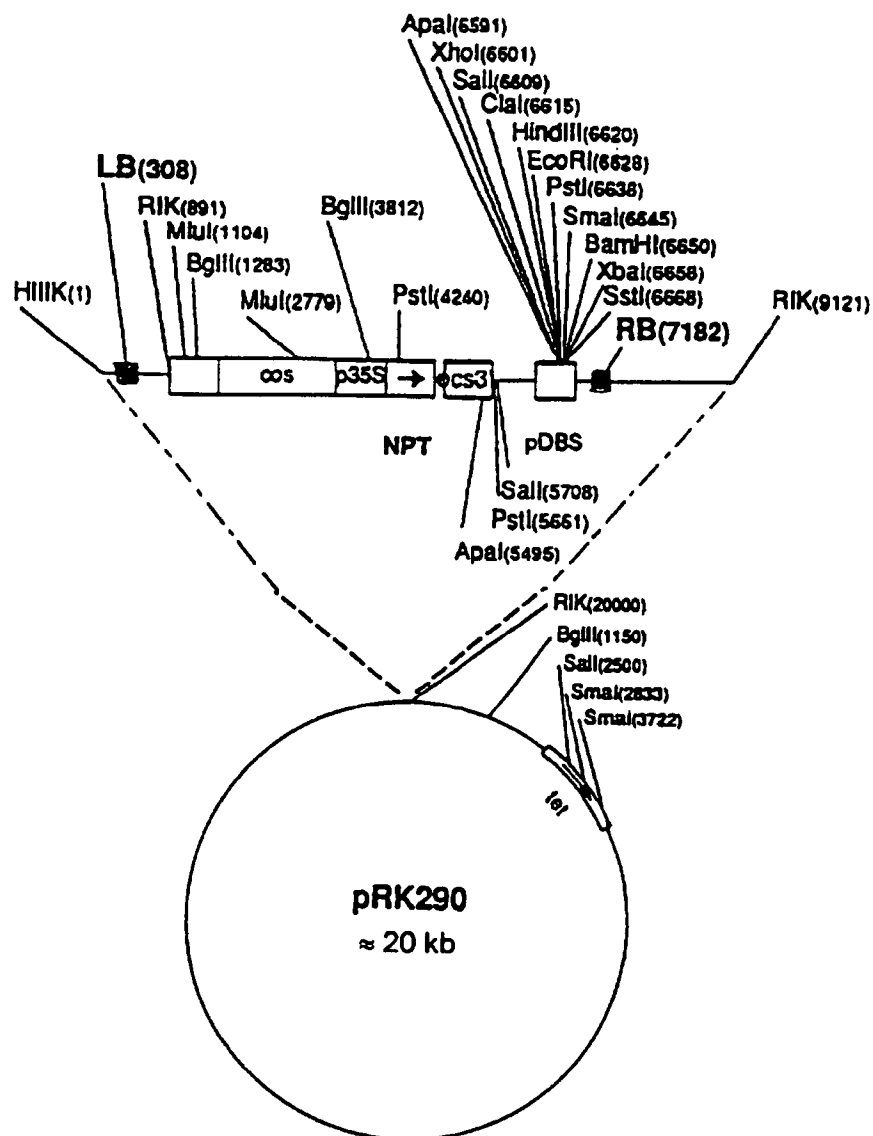


FIGURE 2

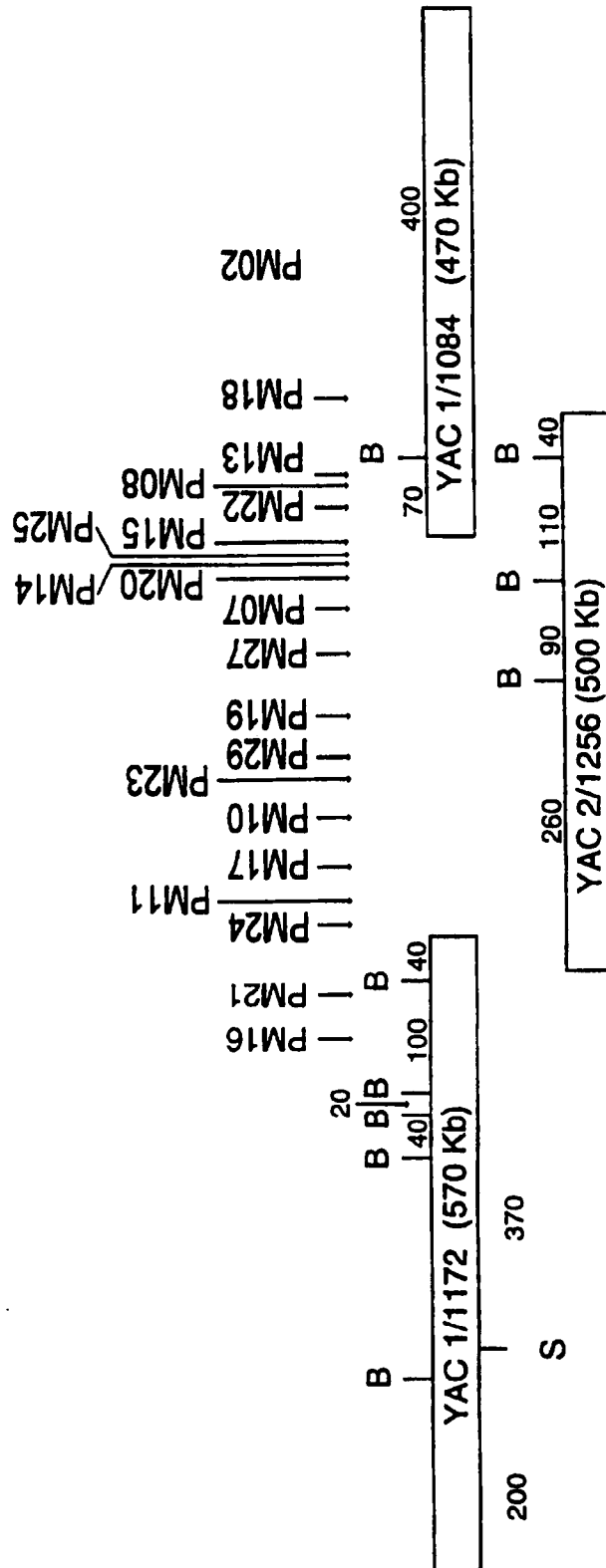


FIGURE 3A

100 Kb
B = BssHII
S = SfiI

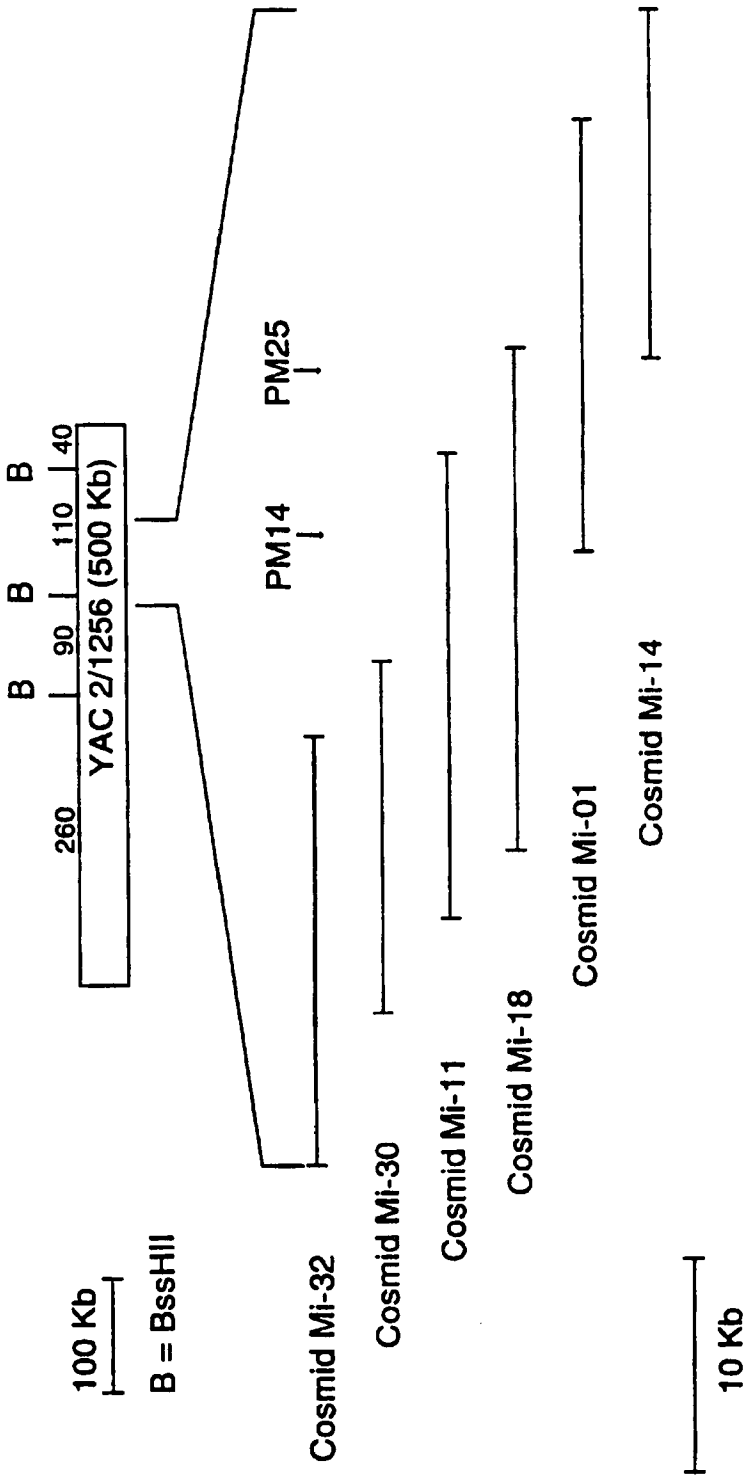
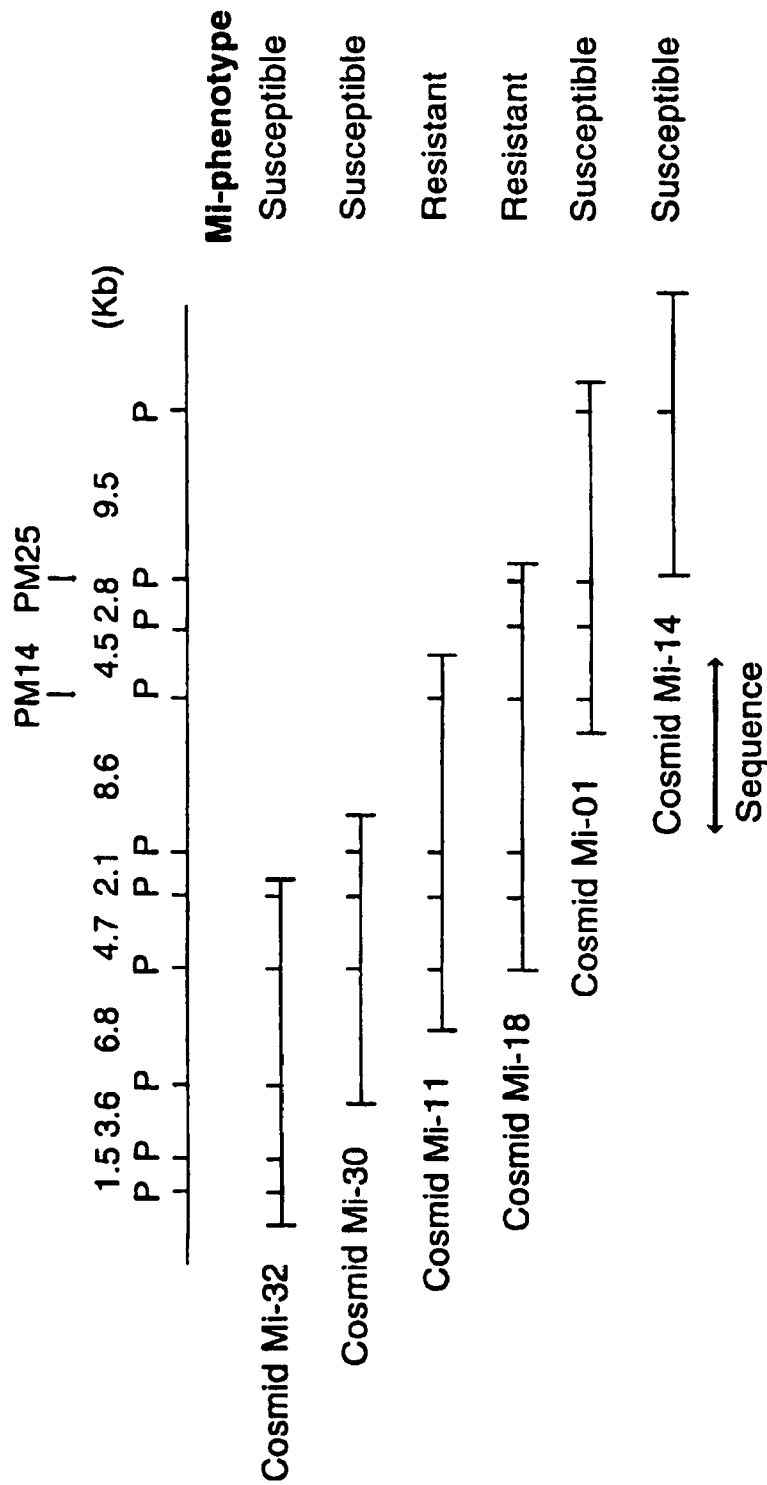


FIGURE 3B



P = Pst I
10 Kb

FIGURE 4

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5' - TTTTCCTCTTCATATAA CTTTTCCTTAACCCCTCTCATGAATAATATAATTGATGTGGA 60
 TAAAGTATTATCCTTTTATGATAAATAACGAAATTTAATAATTTAAAGGGTGCAAATCTAT 120
 AAAATGGAGACGCACATTGATAATGTCCTCTTGATTATTATTAAAGAATTACTCTAGCTT 180
 CACAAATTTAAATTCATTAATGCCTTAATTACATGATAAAAACTTTAGTTGTTCTTTTAC 240
 ATGGTTTGCTAACTTTAATTTTTTTTCTTCATATTCTTCATTTGTTTATTATTATTTTCT 300
 AATTACTTATTTAACTTTTATACTCTTAATATTCATAACTCTCATCTTTTCATATTTCATA 360
 ACCTCCAAATATTTAAACTAAAACCTTTAAGATATCTTTTGATATTTGTTCAATAATAAAT 420
 TCAACTTCTTTATCTTATGAAACCCCTACCAAGATTATTAGGCTATTATTTTTTTATTCTA 480
 TAGTAAAAACAAATGATGAAGATTCTTGAATTTTATAGGATATGAAAGAAGTCGATAAAA 540
 TCTCAGAGAGTTATGTACTAATTTTGTACTTATTTTTTCATCTATATATACATAAATCTT 600
 ATAAGAATAATGTCTATATTGTATTTTTTTTCTTAAATATTATGTTTCTTTTAAATTTTTT 660
 TTCCTCTGTTAGACTTCTTAAATTTAGTTTTCTATGAATGTTTATTTGCCGTAAGTCTTT 720
 GAATTTTGTAATTGTTACATTTTATTATTACATTACGATTTACATATATATTTCCATGAGA 780
 TTTGGTCATTCTAACGTATCTATAAAAATTCACATGAAACACACGTGTGAAGCGCATCCT 840
 CAGAAAACTAGTGTA 900
 TA 960
 TTTTAATCTGGTTAAAAAAGAATAATCTCTTTCCCTTTTTTGACAATATTTAACTTTAAC 1020
 TTTCCACGTAACATGTTTAAAGACAACAAAATTTAAATGACATTTTAATCTTGTAACATAGA 1080
 AAAGTAACATATGATAATTGTCGTTGTCCCTAAACATGATAGATGTATAATTCAAAAGTC 1140
 AATGAATTGTATTTTAGTATTATATTATGAATGAACAAACTGTCAAGATGTGTATATATA 1200
 TATATATTTTATTCTTTGTTAATTTGGCCTTTCAAGTAATTAATTCATTGTTAGGCAGTTG 1260
 AATTAATAATCTCTTTTAGGAATCTTTCCCATGTGAATAACAAGACTTATAAATAAATAA 1320
 TAAAGTCCAGATCTTGTTCATTTGGATCATTTGGCAAACAATTACTCTGTTTCTGAAAC 1380
 AAGGAATAGGGCTTCTAATATTGTAGGGGATTTTTTTTTCTTCATTAATTTATACTTATG 1440
 ATATTAATTATTGTTTTTGAGTACATATTTTAACTCTGTTGTTTATTTTTCTGCAAAGT 1500
 TTCTCCGGTTATATTGAACATATACACATATAGTACATATATTTATTGTAAAAAAAATAA 1560
 TTATTATACTCCATTTCAAGAAATTATGTTTGTATTTATATATATTAATTTCTATAATGTG 1620
 GAAATTGTCAATGTCTACAATGTGTTTGTATGAAATGACAACCACTTGTTTTTATCTGCAA 1680
 CAGTATAAAAATTGGCTTTGCTTCTTTTAGATTAATATAATATTTTACAGGTCACATATT 1740
 ATATTTTATATTGTGAAAGACAAGAGATTTGATTAAAAAAGACTTATGGGTTTGATTTT 1800
 TAATATTTTCAATCTTCTTCAATTAATAAAGACTTTGTATCGTATATTTCACTACTACACT 1860
 TGTTTTCTTATCCAATAGCTTCAACATTATTTCTCAAACAAAGGGTTCTCTAGCTAAACT 1920
 TCAGCCTGTGTAAAGGTAACATCTTCTTTATTCACAGCATAATAACAATGAATTTGGTGC 1980
 ATGTTTGAAGTAAGCTTGAAATTTTCTCTTTCTAAGTTTGTGTTGATCCATTTAGATTCTT 2040
 TTAAATACTTTTGGTATTTAAAGGACTTGTGAAGTCAATGAATTGTATTTTAGTAATCTT 2100
 GCAATTCTAGATCTAGCTATTTGTTGTTCTCTTTTCAACCAAACCTACTTCTTCAATTTGT 2160
 CTAACAAAAATATGTCAAAAAGGTATGAACATGCTTAATCGGAGATCTTTATTGATTCTA 2220
 CTTCACTACTCTAAAAAAAATCTTTTTTCCATTAAGCCCAAGTCGAGATAGGAGAAAA 2280
 ATATTATTAGAGAGATTATTAATTTAATGACATTTTACTCTAGTTTTTTTATCAAAATAAG 2340
 GGAATAATATCCTGTTATTTAACTACCTTTTAAGCATTA TGGGTGGAAAGTAGAAAGAAG 2400
 AAACATAACAGAACAGACAGTAAGTTATGCTTTAATGAGTAGATCTGTATAGGATTACAT 2460
 ATTTGTTTGACTTTTTCGGTGTTCGATTAGAAAACTTACAAGTTTTTAATACATGTATCA 2520
 TTTGTTGATTTGTCCGTTTGGCAGTCATCTGTGGTTACAAGTCACATATGAAGTATGTC 2580
 CACGAGACACACCGAATGTCAAGTATAGATTTCTACTTGATCATACACAACCTTTATCTGA 2640
 GGTTGATGCCAAATTTAAATGACTACCTAAAGCTGATATTTTAAACATTAATCTTGTACA 2700
 CGAAAACTTATTCCTATTACTGTTTTCTTTACCTTTACCTTATAGACTTTTTTGGCAGA 2760
 AAAAAGTTAGACAGATACATTTGATGATGTTTACCATTCTCATTCTCTCTTTATTATT 2820
 TTCTTTACATTCACACGCACAATAATTTCTTGTAGGTTCTTATATGCCATATGCACAT 2880
 AGACGAATCTAGGATTTGATATTTACAAGTTTCTATGTCGACGTCATATTAATATCAATA 2940
 ATAATTAGATTGACAATCACATATTTATAATATTAAGTCGATAACTTTCTTCTTTGTATA 3000
 GGTTGGAAAAGTAATGGTAAACGAGCAGGACTCCTTTTTCTTTTTTTTTGTAAATAATTAA 3060
 CAGTTGTGAGATTTTATGTTTTGTGACTTCATGTCTATAAACATTTTGTGTTGATTAAAGA 3120

FIGURE 5a

TTGACATTTC	CAATTG	TGCGCAGTCT	AAAAATTACTATAT	TGTGAAAAATAGT	GATATATTATTA	GA	3180
TTATTCGTAT	TTTTTTCATCTT	CCTTCTCTCTGTTAAAG	TTTTATCTACTTTTTATTCATCA				3240
GGTCTTGAGAAA	AAGTAGAATCAT	TGGAAAAACGAAAAGATATTGAAGAAGCAAACA	ACTC				3300
		M E K R K D I E E A N N S					13
ATTGGTATGTTAT	TTTTTATAGAGTAAACTGTAAAGTATTGAATTATAGATATGTGGCTTTA						3360
L							14
AAATGTATTAT	TTTTGGCAGGTGTTATTTTTCTGCTCTTAGCAAGGACATTGCCAATGTTCT						3420
		V L F S A L S K D I A N V L					28
AATTTTTCCTAGAGAATGAGGAAAAATCAAAAAGCTCTTTGACAAAGATCAAGTTGAAAAGCT							3480
I F L E N E E N Q K A L D K D Q V E K L							48
AAAATTGAAAATGGCATT	TATTTGTACATATGTTTCAGCTTTCTTATTCCGATTTTGAGCA						3540
K L K M A F I C T Y V Q L S Y S D F E Q							68
GTTTGAAGATATAATGACTAGAAATAGACAAGAGGTTGAGAATCTGCTTCAATCACTTTT							3600
F E D I M T R N R Q E V E N L L Q S L L							88
GGATGATGATGTCCTTACTAGCCTCACCAGTAATATGGATGACTGTATCAGCTTGTATCA							3660
D D D V L T S L T S N M D D C I S L Y H							108
TCGTTCTTATAAATCAGATGCCATCATGATGGATGAGCAATTGGACTTCCTCCTCTTGAA							3720
R S Y K S D A I M M D E Q L D F L L L N							128
TCTGTATCATCTATCCAAGCATCACGCTGAAAAGATATTTCTGGAGTGACTCAATATGA							3780
L Y H L S K H H A E K I F P G V T Q Y E							148
AGTTCTTCAGAATGTATGTGGCAACATAAGAGATTTCATGGGTTGATACTGAATGGTTG							3840
V L Q N V C G N I R D F H G L I L N G C							168
CATTAAGCATGAGATGGTTGAGAATGTCTTACCTCTGTTTCAACTCATGGCTGAAAGAGT							3900
I K H E M V E N V L P L F Q L M A E R V							188
AGGACACTTCCTTTGGGAGGATCAGACTGATGAAGACTCTCGGCTCTCCGAGCTAGATGA							3960
G H F L W E D Q T D E D S R L S E L D E							208
GGATGAACACAATGATAGAGACTCTCGACTCTTCCAGCTAACACATCTACTCTGAAGAT							4020
D E H N D R D S R L F Q L T H L L L K I							228
TGTTCCAAGTGAAGTGGAGGTTATGCACATATGTTTATACAAATTTGAAAGCTTCAACTTC							4080
V P T E L E V M H I C Y T N L K A S T S							248
AGCAGAAGTTGGACGCTTCATTAAGAAGCTCCTGGAAACCTCACCGGATATTCTCAGAGA							4140
A E V G R F I K K L L E T S P D I L R E							268
ATATATCATTCAACTACAAGAGCATATGTTAACTGTTATTCCCCCTAGCACTTTAGGGGC							4200
Y I I Q L Q E H M L T V I P P S T L G A							288
TCGAAACATTATGTCATGATGGAATTCTATTACTTATTCTTTCTGATATGCCCCAAGGA							4260
R N N I H V M M E F L L L I L S D M P K D							308
CTTTATTTCATCATGACAAACTTTTTGATCTCTTGGCTCATGTTGGAACACTTACCAGGGA							4320
F I H H D K L F D L L A H V G T L T R E							328
GGTATCGACTCTTGTACGTGACTTGGAAGAGAAATTAAGGAATAAAGAGGGTAATAACCA							4380
V S T L V R D L E E K L R N K E G N N Q							348
AACAAATTGTGCAACCTAGACTTGCTGGAAATATTGAACTCCTCAAGAAAGATCTCAA							4440
T N C A T L D L L E N I E L L K K D L K							368
ACATGTTTATCTGAAAGCCCCAAATTCATCTCAATGTTGCTTCCCCATGAGTGATGGACC							4500
H V Y L K A C A P N S S Q C C F P M S D G P							388
ACTCTTTCATGCATCTTCTACACATGCACTTAAATGATTTGCTAGATTCTAATGCTTATTC							4560
L F M H L L H M H L N D L L D S N A Y S							408
AATTTCTTTGATAAAGGAAGAAATCGAGTTGGTGAGTCAAGAACTGGAATTCATAAGATC							4620
I S L I K E E I E L V S Q E L E F I R S							428
ATTCTTTGGGGATGCTGCTGAGCAAGGATTGTATAAAGATATCTGGGCACGTGTTCTAGA							4680
F F G D A A E Q G L Y K D I W A R V L D							448
TGTGGCTTATGAGGCAAAAGATGTCATAGATTCAATTATTGTTCCGAGATAATGGTCTCTT							4740
V A Y E A K D V I D S I I V R D N G L L							468
ACATCTTATTTTCTCACTTCCCATTACCATAAAGAAGATCAAACCTTATCAAAGAAGAGAT							4800

FIGURE 5b

CTCTGCTTTAGATGAGAACATTCCCAAGGACAGAGGTCTAATCGTTGTGAACCTCTCCCAA 4860
 S A L D E N I P K D R G L I V V N S P K 508
 GAAACCAGTTGAGAGAAAGTCATTGACAACCTGATAAAATAATTGTAGGTTTTGAGGAGGA 4920
 K P V E R K S L T T D K I I V G F E E E 528
 GACAAACTTGATACTTAGAAAGCTCACCAGTGGACCCGAGATTTAGATGTCATTTTCGAT 4980
 T N L I L R K L T S G P A D L D V I S I 548
 CACCGGTATGCCGGGTTTCAGGTAAACTACTTTGGCATACAAAAGTATACAATGATAAGTC 5040
 T G M P G S G K T T L A Y K V Y N D K S 568
 AGTTTCTAGACATTTTGACCTTCGTGCATGGTGCACGGTCGATCAAGGATATGACGACAA 5100
 V S R H F D L R A W C T V D Q G Y D D K 588
 GAAGTTGTTGGATACAATTTTCAGTCAAGTTAGTGGCTCAGATTCAAATTTGAGTGAGAA 5160
 K L L D T I F S Q V S G S D S N L S E N 608
 TATTGATGTTGCTGATAAATTGCGGAAACAACCTGTTTGGAAAGAGGTATCTTATTGTCTT 5220
 I D V A D K L R K Q L F G K R Y L I V L 628
 AGATGATGTGTGGGATACTACTACATTGGATGAGTTGACAAGACCTTTTCCTGAAGCTAA 5280
 D D V W D T T T L D E L T R P F P E A K 648
 GAAAGGAAGTAGGATTATTTTGACAACCTCGAGAAAAGGAAGTGCTTTGCATGGAAAGCT 5340
 K G S R I I L T T R E K E V A L H G K L 668
 GAACACTGATCCTCTTGACCTTCGATTGCTAAGACCAGATGAAAGTTGGGAACCTTTTAGA 5400
 N T D P L R L R L R P D E S W E L L E 688
 GAAAAGGACATTTGGTAATGAGAGTTGCCCTGATGAAGTATTAGATGTCGGTAAAGAAAT 5460
 K R T F G N E S C P D E L L D V G K E I 708
 AGCCGAAAATTGTAAAGGGCTTCCTTTGGTGGCTGATCTGATTGCTGGAGTCATTGCTGG 5520
 A E N C K G L P L V A D L I A G V I A G 728
 GAGGGAAAAGAAAAGGAGTGTGTGGCTTGAAGTTCAAAGTAGTTTGAGTTCTTTTATTTT 5580
 R E K K R S V W L E V Q S S L S S F I L 748
 GAACAGTGAAGTGGAAGTGATGAAAGTTATAGAATTAAGTTATGACCATTTACCACATCA 5640
 N S E V E V M K V I E L S Y D H L P H H 768
 CCTCAAGCCATGCTTGCTTCACTTTGCAAGTTGGCCGAAGGACACTCCTTTGACAATCTA 5700
 L K P C L L H F A S W P K D T P L I Y 788
 TTTGTTGACTGTTTATTTGGGTGCTGAAGGATTTGTGGAAAAGACGGAGATGAAGGGTAT 5760
 L L T V Y L G A E G F V E K T E M K G I 808
 AGAAGAAGTGGTGAAGATTTATATGGATGATTTAATTTCCAGTAGCTTGGTAATTTGTTT 5820
 E E V V K I Y M D D L I S S S L V I C F 828
 CAATGAGATAGGTGATATACTGAATTTCCAAATTCATGATCTTGTGCATGACTTTTGTTT 5880
 N E I G D I L N F Q I H D L V H D F C L 848
 GATAAAAGCAAGAAAGGAAAATTTGTTTGATCGGATAAGATCAAGTGCTCCATCAGATTT 5940
 I K A R K E N L F D R I R S S A P S D L 868
 GTTGCCTCGTCAAATTACCATTGATTATGATGAGGAGGAGGAGCACTTTGGGCTTAATTT 6000
 L P R Q I T I D Y D E E E E H F G L N F 888
 TGTCATGTTTCGATTCAAATAAGAAAAGGCATTCTGGTAAACACCTCTATTCTTTGAGGAT 6060
 V M F D S N K K R H S G K H L Y S L R I 908
 AAATGGAGACCAGCTGGATGACAGTGTTCCTGATGCATTTTCACCTAAGACACTTGAGGCT 6120
 N G D Q L D D S V S D A F H L R H L R L 928
 TATTAGAGTGTGGACCTGGAACCTCTTTAATCATGGTGAATGATTCTTTGCTGAATGA 6180
 I R V L D L E P S L I M V N D S L L N E 948
 AATATGCATGTTGAATCATTTGAGGTACTTAAAGAATTCGGACACAAGTTAAATATCTGCC 6240
 I C M L N H L R Y L R I R T Q V K Y L P 968
 TTTCTCTTTCTCAAACCTCTGGAATCTAGAAAGTCTGTTTGTGTCTAACAAAGGATCAAT 6300
 F S F S N L W N L E S L F V S N K G S I 988
 CTTGGTACTATTACCGAGAAATTTGGATCTTGTAAGTTGCGAGTGCTGTCCGTGGGTGC 6360
 L V L L P R I L D L V K L R V L S V G A 1008

FIGURE 5c

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TTGTTCTTTCTTTGATATGGATGCAGATGAATCAATATTGATAGCAAAGGACACAAAGTT 6420
 C S F F D M D A D E S I L I A K D T K L 1028
 AGAGAAGTTGAGAATATTAGGGGAAGTGTGATTTTCTATTTCGAAAGATACAAATGAATAT 6480
 E N L R I L G E L L I S Y S K D T M N I 1048
 TTTCAAAGGTTTCCCAATCTTCAGGTGCTTCAGTTTGAAGTCAAGGAGTCATGGGATTA 6540
 F K R F P N L Q V L Q F E L K E S W D Y 1068
 TTCAACAGAGCAACATTGGTTCCCGAAATTGGATTGCCTAACTGAACTAGAAACACTCTG 6600
 S T E Q H W F P K L D C L T E L E T L C 1088
 TGTAGGTTTTTAAAAGTTCAAACACAAACCACTGTGGGTCTCTGTTGCGACAAATCGGCC 6660
 V G F K S S N T N H C G S S V A T N R P 1108
 GTGGGATTTTCACTTCCCTTCAAATTTGAAAGAACTGTTGTTGTATGACTTTCTCTGAC 6720
 W D F H F P S N L K E L L Y D F P L T 1128
 ATCCGATTCACATCAACAATAGCGAGAGTGCCTAACCTTGAAAATTTGTCCCTTTATGA 6780
 S D S L S T I A R L P N L E N L S L Y D 1148
 TACAATCATCCAGGGAGAAGAAATGGAACATGGGGGAGGAAGACACTTTTGAGAATCTCAA 6840
 T I I Q G E E W N M G E E D T F E N L K 1168
 ATTTTTGAAGTTGCGTCTACTGACTCTTTCCAAGTGGGAGGTTGGAGAGGAATCCTTCCC 6900
 F L N L R L L T L S K W E V G E E S F P 1188
 CAATCTTGAGAAATTAAGAACTGCAGGAATGTGGTAAGCTTGAGGAGATTCCACCTAGTTT 6960
 N L E K L K L Q E C G K L E E I P P S F 1208
 TGGAGATATTTATTCATTGAAATTTATCAAAATTGTAAAGAGTCTCAACTTGAAGATTC 7020
 G D I Y S L K F I K I V K S P Q L E D S 1228
 TGCTCTCAAGATTAGAAATACGCTGAAGATATGAGAGGAGGGAACGAGCTTCAGATCCT 7080
 A L K I K K Y A E D M R G G N E L Q I L 1248
 TGGCCAGAAGAATATCCCCTTATTTAAGTAGCATTGTTGGTTGAAGTTTGTCTGGTGATAT 7140
 G Q K N I P L F K --- 1257
 TGTATATGATTAATAATATCCTGTGATGAGATTCCTCTTAGTTTCTTTTAAACAAAAATAT 7200
 AATTTTTATAAGTACACATATCGTTTGTTAATTTGTCCATTTGTGATTGCAAGTCACACA 7260
 TGAGGTATGTTTCGTATTATGGGTTTCAACTTGATCAGACGTAATTTAAGATAAGTGCTT 7320
 ATATGATGTTGCATGCCAGATGGAAGTGACTATGTGAAGTTTATATTTTAAACATTAATC 7380
 TTGTATACCAAATACTATTCCTATGCTATGTTGTTTGGCATTGTGCTTCTCTCTTTATT 7440
 TTTTTTCTTTCCATTCACACACACATTAATTTCTAGTAGACCGCATATTACTACATCTG 7500
 TATTGTCCGTATACAAGACGAATCCAGGATTGATGTTTACAAGTATTTGTGAAGAATCC 7560
 AGGATTTGATGTTTACAAGACAATTAGATTCTATATGTATAGGATTTTGACAGAACTG 7620
 AGGGATTACATGACAATTACTCTGTGGATTGCTTTGGCTGTCCAAACCTCCTTTGTG 7680
 TCTAACTTCGTCTGAAGTCCCATTTATATGCTCAAAGCTCAGTCAAGGTACTGATTCAA 7740
 AGCTAGGCTGTGAAGTAACTTTTAAATGATATTGCTGCAAAGTCGCTCAACAAAGGGTC 7800
 ATAACCATCACTACAATACTACAAGCTCAAGCAAGTAAACGCGGGTGAAAGATTAACATA 7860
 GATCGCTATCCCCTGCAAAAGCTAAGGAAAGCATCTCTAACTTCTTAGCATGTACTCAA 7920
 CACACGATCTGTAAGGATGCCAGAAAGAGAAAGTTACGTTGCCGCAATTCCCTTACAGTGT 7980
 TGCACAATGTCCCCAAAACCAATCACACTACAAAAAAGGCTCAAATTTCTGGGGGTTA 8040
 TAATTAGACGGTCAATAACCCCTGCAATTTAGTGTGTTGGAGGTGAATAAACTCCTCCA 8100
 ATTAGGAGTGTACAAATTAAGTCGCGTGGGATTCTTGGCACATCCCGGTAAGGTTAACTA 8160
 GCGGGGGTTTGAACCCCAACCGCATTTCAAAGTAGGAGTCGAAACCCCAACGATTTGTG 8220
 AACTCGGGGGAGTCAAAAAACCCCGCAATAAATGATTTTACATTAAAAATTAATAGGAGC 8280
 TTGGACCCCTGTGATTTATGAAATATAACTTTTTGTAGCATTTGCCAGAAATATCAATT 8340
 TTAGATACTAATAATAAATTAATTAACATAACATGTGCATCATTATTCAAAGGACATATTA 8400
 GTATTAAGAAATAATACAATATTCAACACAAAAGTACCCAAACTCAAGATAGGATCAGTT 8460
 TATGGAAGTTCAACTAGTTTCACTATAATTATGTCACTAACATCAGCTGGCTGCAAAGG 8520
 AGAATACATAATAAGTGACTTTATCCAACTCAAAATCATGGCTGAATGTAGTAAACAC 8580
 CAAAGATTATAAATTTCCATTAATTATCATATACATACACAACAACAACTTAAACAA 8640
 TATAGAAAAGGATTAAACCATTTACACAAGCAATGATTCTATACCATTTCAAAACGACAA 8700
 CATACTGTACTACTAAACAAGACACCATCAAAGTATTTGGACAAATATTAACAATAGTT 8760

FIGURE 5d

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AAAACATGAACAAAGAATCTC⁵AGGTTTCTTGTTCAGTAGAAAAGAGACAGACTAGGAACTG 8820
GAGTGCTATTTTTCTTATAAGAGACAATTAATGTTTACTTCTTTATATTTTGACTATAAG 8880
TTGATTGGTTATAATGTTTACGAGGTTGTATATAATCCGATGTTCAATGATATGACTTTC 8940
CTATTGACTGAAATGCTTGAACGCAAACAGTATATCTAGATTAAGAATGAGGACGAATTA 9000
CCTCTAGAGGCATGGGTAATGGAAGCATAACTCCTTGATAATGGTTGT¹⁰TAGCCCACTGCA 9060
AGTCACAAAACAAAACATCCGTAATATTAACATACTAAGGTTGTAAGCACTAAACGACAA 9120
CAACTATGCCTCAATCCCAACTAAGTTGGAATCGACTATATGAATACTCACAATTTTCGAT 9180
TTATAGACAAAGATACTAGTAGAAATGACGTCCTTTCCTTTCTATGTTAACACTTGGACAG 9240
AGAATGTTAAAGACTTACAACAACAGAAAAGAGTTAAATCATTTAATTGAGCAAGGATT 9300
TCAAAACGACAACACAATATACTCAATTTTTTCGACGGAAACAAC¹⁵TGGTTGGACAACAGTG 9360
CTATTTGTAACTCCAATGAACAACACTGCAACGTACATGTATCTCATTGCACTAAATAAA 9420
TCCCGTTGAGAGTAACATATCAATAGTTACGAACAATATGATCACGACAAAGGATTGTAA 9480
GTACCACAGGACAAGTCATGCTTGCATGAAAAACGGATATGTAAAGAACCAAAATCCTGC 9540
TGCTGAAATAAGCAGTTATGATTATCCAAAAATCATGAATACACATGCAC²⁰TGAGTTTGT 9600
TCCAAGAAAAACACAACCAACTACTGTGCAAGTGAAGATTCAAAAGTGACTATTGATGT 9660
TAATTCTTCCACAAGGTTGAATAATTTTGTCACTATAGGATTTAAGACGAAGAAGAAACA 9720
GGCGACAATTTTGTAAGCATAGACCTTCTTATGCAACTATGAGCTGGTATGCTATTCATT 9780
TTCTTTTACTCGTAAAAATCGTTGATACTAAAGAATGCCAATCCAGTCCTGCTGAATAGGC 9840
GCCAGGTGACTGGTTGCTGTTAATAATTTT-3' 9870

FIGURE 5e

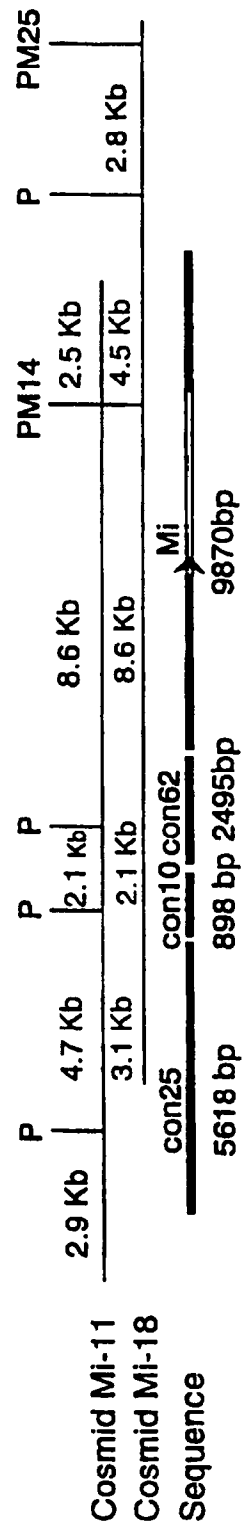
[illegible]

FIGURE 6

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MEKRKDIEEA NNSLVLFSA SKDIANVLIF LENEENQKAL DKDQVEKLKL KMAFICTYVQ
LSYSDFEQFE DIMTRNRQEV ENLLQSLDD DVLTSLTSNM DDCISLYHRS YKSDAIMMDE
QLDFLLNLNLY HLSKHHAKEI FPGVTQYEV LQNVCGNIRDF HGLILNGCIK HEMVENVLPL
FQLMAERVGH FLWEDQTDDE SRLSELDEDE HNDRDSRLFQ LTHLLLKIVP TELEVMHICY
TNLKASTSAE VGRFIKKLE TSPDILREYI IQLQEHMLTV IPPSTLGARN IHVMMEFLLL
ILSDMPKDFI HHDKLFDLLA HVGTLTREVS TLVRDLEEK RNKEGNNQTN CATLDLLENI
ELLKKDLKHV YLKAPNSSQC CFPMSDGPLF MHLHMLND LLDNAYSIS LIKEEIELVS
QELEFIRSFF GDAAEQGLYK DIWARVLDVA YEAKVIDSI IVRDNGLLHL IFSLPITIKK
IKLIKEEISA LDENIPKDRG LIVVNSPKKP VERKSLTDDK IIVGFEEETN LILRKLTSGP
ADLDVISITG MPGSGKTTLA YKVYNDKSVS RHFDLRAWCT VDQGYDDKKL LDTIFSQVSG
SDSNLSENID VADKLKQLF GKRYLIVLDD VWDTTTLDL TRPFPEAKKG SRIILTREK
EVALHGKLN DPLDLRLLRP DESWELLEKR TFGNECPDE LLDVGKEIAE NCKGLPLVAD
LIAGVIAGRE KKR SVWLEVQ SSLSSFILNS EVEVMKVIEL SYDHLPHHLK PCLLHFASWP
KDTPLTIYLL TVYLGAEFV EKTEMKGIEE VVKIYMDDLI SSSLVICFNE IGDILNFQIH
DLVHDFCLIK ARKENLFDRI RSSAPSDLLP RQITIDYDEE EEHFGLNFVM FDSNKKRHSG
KHLVSLRING DQLDDSVSDA FHLRHLRLIR VLDLEPSLIM VNDLLNEIC MLNHLRYLRI
RTQVKYLPFS FSNLWNLESL FVSNKGSILV LLPRILDLVK LRVLSVGACS FFDMDADESI
LIAKDTKLEN LRILGELLIS YSKDTMNIFK RFPNLQVLQF ELKESWDYST EQHWFPKLDC
LTELETLCVG FKSSNTNHCG SSVATNRPWD FHFPNLKEL LLYDFPLTSD SLSTIARLPN
LENLSLYDTI IQGEEWNMGE EDTFENLKF LNRLLTSLKW EVGEESFPNL EKLKLQECGK
LEEIPPSFGD IYSLKFIKIV KSPQLEDSAL KIKKYAEDMR GGNELQILGQ KNIPLFK
//

FIGURE 7A

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MAFICTYVQL SYSDFEQFED IMTRNRQEVE NLLQSLDDDD VLTSLSNMD DCISLYHRSY
KSDAIMMDEQ LDFLLLNLYH LSKHHA EKIF PGVTQYEV LQ NVCGNIRDFH GLILNGCIKH
EMVENVLPLF QLM AERVGHF LWEDQTD EDS RLSE LDEDEH NDRDSRLFQL THLLLKIVPT
ELEV MHICYT NLKASTSAEV GRFIKKLLET SPDILREYII QLQEHMLTVI PPSTLGARNI
HVMMEFLLLI LSDMPKDFIH HDKLFDLLAH VGTLTREVST LVRDLEEKLR NKEGNNQTN C
ATLDLLENIE LLKKDLKHVY LKAPNSSQCC FPMSDGPLFM HLLHMH LNDL LDSNAYSISL
IKEEIELVSQ ELEFIRSFFG DAAEQGLYKD IWARVLDVAY EAKDVIDSII VRDNGLLHLI
FSLPITIKKI KLIKEEISAL DENIPKDRGL IVVNSPKKPV ERKSLTTDKI IVGFEEETNL
ILRKLTSGPA DLDVISITGM PGSGKTTLAY KVYNDKSVSR HFDLRAWCTV DQGYDDKKLL
DTIFSQVSGS DSNLSENIDV ADKLRKQLFG KRYLIVLDDV WDTTTLDEL T RPFPEAKKGS
RIILTTREKE VALHGKLNTD PLDLRLLRPD ESWELLEKRT FGNE SCPDEL LDVGKEIAEN
CKGLPLVADL IAGVIAGREK KRSVWLEVQS SLSSFILNSE VEV MKVIELS YDHLPHHLKP
CLLHFASWPK DTPLTIYLLT VYLGAEGFVE KTEMKGIEEV VKIYMDDLIS SSLVICFNEI
GDILNFQIHD LVHDFCLIKA RKENLFDRI R SSAPSDLLPR QITIDYDEEE EHFG LNFVMF
DSNKKRHSGK HLYSLRINGD QLDDSVSDAF HLRHLRLIRV LDLEPSLIMV NDSLLNEICM
LNHLRYLRIR TQVKYLPFSF SNLWNLESF VSNKGSILVL LPRILDV LKLV RVL SVGACSF
FDMDADESIL IAKDTKLENL RILGELLISY SKDTMNIFKR FPNLQVLQFE LKESWDYSTE
QHWFPKLDCL TELETLCVGF KSSNTNHCGS SVATNRPWDF HFPSNLKELL LYDFPLTSDS
LSTIARLPNL ENLSLYDTII QGEEWNMGEE DTFENLKFLN LRLLT LSKWE VGEESFPNLE
KLKLQECGKL EEIPPSFGDI YSLKFIKIVK SPQLEDSALK IKKYAEDMRG GNELQILGQK
NIPLFK
//

FIGURE 7B

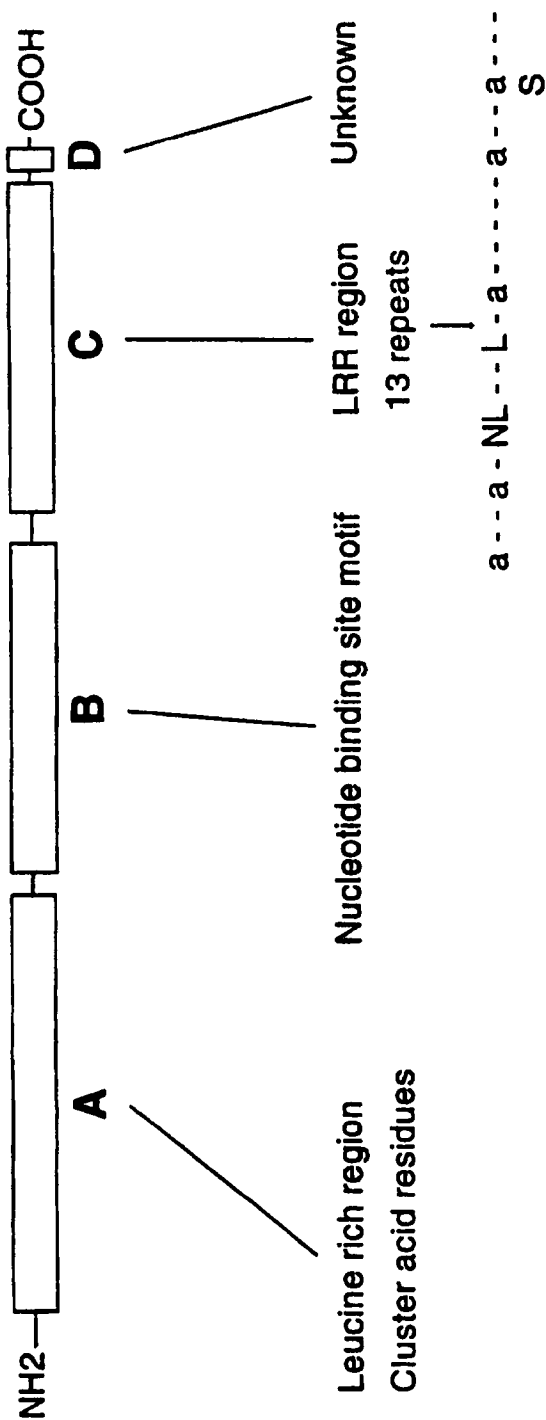


FIGURE 8

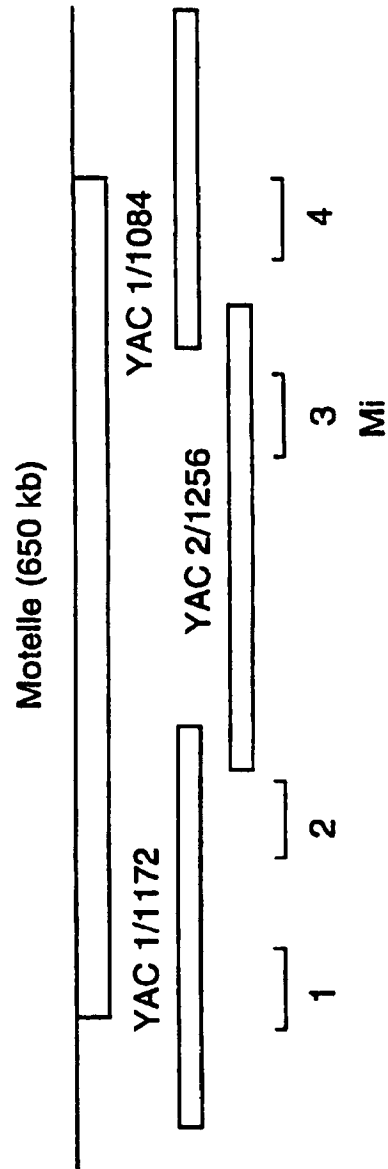


FIGURE 9